Regulation of Kinin Receptor Expression in Heart Failure with a Focus on Vascular Endothelium

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Academic Dissertation

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MAIN ABBREVIATIONS

ACE	=	Angiotensin converting enzyme 1
ACEI	=	Angiotensin converting enzyme inhibitor
ANG II	=	Angiotensin II
APM	=	Aminopeptidase M
ANP	=	Atrial natriuretic peptide
APP	=	Aminopeptidase P
ARB	=	Angiotensin receptor 1 inhibitor
AT	=	Angiotensin
BNP	=	Brain-type natriuretic peptide
cGMP	=	Cyclic guanosine monophosphate
CHD	=	Coronary heart disease
CNP	=	C-type natriuretic peptide
CPM	=	Carboxypeptidase M
CPN	=	Carboxypeptidase N
COX	=	Cyclooxygenase
COXIB	=	Cox-enzyme inhibitor
CTGF	=	Connective tissue factor
EDHF	=	Endothelium derived hyperpolarizing factor
EC	=	Endothelial cell
FGF	=	Fibroblast growth factor
HF	=	Heart failure
HIF	=	Hypoxia inducing factor
HMG-CoA	=	3-hydroxy-3-methylglutaryl-coenzyme A
HMWK	=	High molecular weight kininogen
HTk	=	Human tissue kallikrein
HUVEC	=	Human umbilical vein endothelial cell
IDC	=	Idiopathic dilating cardiomyopathy
IFN-γ	=	Interferon gamma
IL-1β	=	Interleukin 1- beta
IL-2	=	Interleukin 2
IL-10	=	Interleukin 10
LMWK	=	Low molecular weight kininogen
LPS	=	Lipopolysaccaride
LVH	=	Left ventricular hypertrophy
KO	=	Knockout
MAPK	=	Mitogen-activated protein kinase
MVEC	=	Microvascular endothelial cell
NA	=	Noradrenaline

NEP	=	Neutral endopeptidase
NOS	=	Nitric oxide synthase
NSAID	=	Non-steroidal anti-inflammatory drug
RAS	=	Renin-angiotensin-system
RCMEC	=	Rat coronary microvascular endothelial cell
SMC	=	Smooth muscle cell
SNP	=	Single nucleotide polymorphism
TNF-α	=	Tumor necrosis factor–alpha
VEGF	=	Vascular endothelial growth factor

ABSTRACT

Accumulating evidence, in human and experimental animal models, show that kinins, notably bradykinin (BK) and kallidin, have cardioprotective effects. To these include reduction of left ventricular hypertrophy (LVH) and progression of heart failure. Their effects are mediated through two G protein-coupled receptors- bradykinin type-2 receptor (BK-2R) and bradykinin type-1 receptor (BK-1R). The cardioprotective effects of BK-receptors relate to triggering the production and release of vasodilating nitric oxide (NO) by endothelial cells. They also exert anti-proliferative effects on fibroblasts and anti-hypertrophic effects on myocytes, and thus may play an essential role in the cardioprotective response to myocardial injury. The cardioprotective role of BK-2Rs is widely accepted. The role for BK-1Rs in HF, is based on experimental animal models, where the receptors have been linked to cardioprotective- but also to cardiotoxic -effects. The BK-1Rs are induced under inflammatory and ischemic conditions. The induction has been shown in animal models; no previous reports, concerning BK-1Rs in human heart failure, have been presented.

Previously, it has been shown, that the expression of cardioprotective BK-2Rs were down-regulated in human end-stage heart failure. Present results showed that, in end-stage heart failure patients, the BK-1Rs were up-regulated, suggesting that also BK-1Rs may be involved in the pathogenesis of human heart failure. The receptors were localized mainly in the endothelium of the intramyocardial coronary vessels, and correlated with the increased TNF-α expression in the myocardial coronary vessels. Moreover, in cultured endothelial cells, it was shown, that TNF-α is a potent trigger of BK-1Rs. These results suggest, that cytokines, such as TNF-α may be responsible for the up-regulation of BK-1Rs in human heart failure.

A linear relationship was discovered between BK-2R mRNA and protein expression in normal and failing human left ventricles, implying that the BK-2Rs are regulated on the transcriptional level, at least in human myocardium. The expression of BK-2Rs correlated positively with age in normal and dilated hearts (IDC), though the increase in receptors was clearly lower in dilated hearts. The results suggest, that human hearts adapts to age-related changes, by up-regulating the expression of cardioprotective BK-2Rs. It was also presented, that in the promoter polymorphism -58 T/C, the C-allele was accumulated in cardiomyopathy patients which may partially explain the reduced number of BK-2Rs, in these patients. The BK-receptors seem to contribute to the pathogenesis of human heart failure and they localize mainly in the endothelium of the myocardial coronary vessels.

Statins are cardioprotective drugs, which reduce the level of plasma cholesterol, but also exert several non-cholesterol-dependent effects. The effects of statins were studied in human coronary arterial endothelial cells (hCAEC) and incubation with lovastatin induced both BK-1 and BK-2Rs in a time and concentration-dependent way. Moreover, the induced BK-2Rs were functionally active, thus NO production and cGMP signaling was increased. The induction was abrogated by mevalonate, a direct HMG-CoA metabolite. Lovastatin is known to inhibit Rho activation, and by a selective RhoA kinase inhibitor Y27632, we showed a similar induction of BK-2R expression as with lovastatin. Interestingly a COX-2-inhibitor NS398 inhibited this lovastatin-induction of BK-2Rs, suggesting that COX-2 inhibitors may affect the endothelial BK-2Rs, in a negative fashion.

Hypoxia is a common denominator in HF but also in other cardiovascular diseases. An induction of BK-2Rs in mild hypoxic conditions was shown in cultured hCAECs, which was abolished by a specific BK-2R inhibitor Icatibant. These receptors were functionally active, thus BK increased and Icatibant inhibited the production of NO. In rat myocardium the expression of BK-2R was clearly increased in the endothelium of vessels, forming at the border zone, between the scar tissue and the healthy myocardium. Moreover, in *in vitro* wound-healing assay, endothelial cells were cultured under hypoxic conditions and BK significantly increased the migration of these cells and as Icatibant inhibited it. These results show, that mild hypoxia triggers a temporal expression of functionally active BK-2Rs in human and rat endothelial cells, supporting a role for BK-2Rs, in hypoxia induced angiogenesis.

Our and previous results show, that BK-Rs have an impact on the cardiovascular diseases. In humans, at the end stage of heart failure, the cardioprotective BK-2Rs are down-regulated and BK-1Rs induced. Whether the up-regulation of BK-1Rs, is a compensatory mechanism against the down-regulation of BK-2Rs, or merely reflects the end point of heart failure, remains to bee seen. In a clinical point of view, the up-regulation of BK-2Rs, under hypoxic conditions or statin treatment, suggests that, the induction of BK-2Rs is protective in cardiovascular pathologies and those treatments activating BK-2Rs, might give additional tools in treating heart failure.

REVIEW OF THE LITERATURE

1. Heart Failure

1.1 Heart Failure – General

Heart failure (HF) is traditionally viewed as a hemodynamic syndrome characterized by fluid retention, high venous pressure, and low cardiac output. It is usually, but not always, caused by a defect in myocardial contraction – myocardial failure – and it is a principal complication of virtually all forms of heart disease (93). The failing heart presents abnormal growth responses – namely myocyte hypertrophy, progressive remodeling and increased myocardial fibrosis. These structural abnormalities, which now are recognized to play a central role in determining the poor prognosis in this clinical syndrome, result from changes in the size, shape, and molecular composition of both cardiac myocytes and non-myocytes (221).

The major causes of HF are coronary heart disease (CHD), hypertension, and valvular diseases, which account for over 80% of the underlying causes. Other causes include entities such as diseases of the myocardium (myocarditis, cardiomyopathies) and pericardium, diseases of the lung and pulmonary vasculature, arrhythmias, and other diseases such as hyperthyreosis, alcohol abuse and diabetes.

During the last decades the understanding and treatment of heart failure has changed dramatically. Large, randomized clinical trials in the 1980s revealed the poor prognosis of HF patients and changed the view of HF being merely a hemodynamic disorder (220). It has now been realized that changes in the structure and composition of the myocardium play a key role in determining the survival of these patients (220). Patients often remain asymptomatic or minimally symptomatic after initial pump dysfunction has been present for many years. The progression of HF is related to the progression of underlying diseases, neurohumoral abnormalities, and LV remodeling (93).

Heart failure is an increasing cause of morbidity and mortality in the Western world (15). Despite improved treatment and a relative reduction in mortality, the prognosis remains poor. The 5-year survival rate of all HF patients is only 50%, and it is even worse in the more severe cases (15).

The prevalence of heart failure in Europe varies between 0.4% and 2.0% (95). It increases dramatically with age, being 1-2% for persons aged 50-59 years and 6-8% for individuals over 75 years old (93). The mean age of the heart failure population in Europe is 74 years (485). Thus, HF is the leading hospital discharge diagnosis in patients over 65 years old and the prevalence is increasing, which is shown by the 55% increase in the number of hospitalizations

due to HF between 1985 and 1995 (93). Fifteen million individuals in Europe and about five million in America (2.2% of the population) currently have HF and 550 000 patients are diagnosed with HF annually (138;420).

The clinical manifestations of heart failure depend on the rate at which the syndrome develops and specifically on whether sufficient time has elapsed for compensatory mechanisms to develop. Chronic HF can be described as a progressive disease where acute HF episodes with repetitive hospital admissions disrupt the normal progression of the disease.

Since the Framingham study, there have been efforts to improve the prognosis of HF and the treatments have indeed improved. Before ACE inhibitors, the treatment of HF consisted of merely relieving the symptoms with diuretics and nitrates. ACE inhibitors were the first to actually have an impact on the symptoms, but more important on morbidity and mortality. In the last ten years the treatment of heart failure has advanced and whole new categories of drugs have been developed. Despite these new drugs mortality has not diminished dramatically.

1.2 Pathogenesis of Heart Failure

When the heart has difficulty pumping blood at a rate commensurate with the requirements of metabolizing tissues or does so only by elevating the filling pressure, heart failure develops. At the same time the heart switches on a number of adaptive mechanisms for maintaining its pumping function (93). Essential among the adaptive systems are the Frank-Starling mechanism, activation of the neurohumoral systems and myocardial remodeling.

Increased preload helps to sustain the cardiac performance: reduced cardiac output activates neurohumoral responses, leading to vasoconstriction, fluid retention, and cardiac stimulation (93). These adaptations occur rapidly and may be adequate to maintain pumping performance for a short time. Myocardial hypertrophy and progressive dilation (remodeling) of the failing heart account for abnormal proliferative responses and play an important role in long-term adaptation to hemodynamic overload. The structural responses play a crucial role in the poor prognosis of HF, which results from changes in the shape, size, and molecular composition of both cardiac myocytes and other cardiac cells (219).

The development of HF is generally progressive where the initial step challenges cardiac performance- either systolic or diastolic function. Regardless of the initial stimulus, adaptive, compensatory mechanisms are activated in both the heart and the circulatory system. These include activation of the neurohumoral pathway, specifically the renin-angiotensin-aldosterone (RAA) system, and the sympathetic nervous system. This activation leads to production of several neurohormones, which have both hemodynamical

and biological effects on the myocardium and the systemic vascular bed. At first these mechanisms are compensatory, protecting the heart by balancing cardiac output with demands. As the deleterious activation is prolonged the compensatory mechanisms become noxious to the myocardium, leading to alteration of the existing structures (93).

The activation of adaptive mechanisms also leads to the development and progression of LV dysfunction and ultimately to a clinical syndrome of HF (93).

The purpose of these adaptive mechanisms is to sustain cardiac performance in spite of the changed hemodynamic conditions.

1.3 Remodeling → Heart Failure

Left ventricular remodeling is a compensatory process in which mechanical, neuro- hormonal, and possibly genetic factors contribute to alteration of ventricular size, shape, and function (93). It develops over months and years and is crucial to hemodynamic adaptation. It is also irreversible and initially adaptive, thus finally leading to left ventricular hypertrophy (LVH) and clinical HF.

Two major components characterize LVH: hypertrophy of the myocytes and myocardial fibrosis, mainly located in the interstitium but also in the perivascular areas of the myocardium (347;497).

LVH initially compensates for volume or pressure overload by increasing either the size of the left ventricle or its capacity to produce force. Finally, LVH impairs the diastolic function by increasing the stiffness of the left ventricle, working consecutively against the active phase of relaxation and atrial filling. Later, the stiffness produced by LVH begins to interfere with contraction, thereby also affecting the systolic function of the heart.

LVH is associated with ischemia, arrhythmias, and heart failure and represents an independent risk factor of cardiovascular mortality (305). The transition from compensated hypertrophy to manifested HF involves a complex of events at the cellular and molecular level – hypertrophy of cardiac myocytes, changes in gene expression with re-expression of fetal programs and decreased expression of adult programs, changes in the nature and quantity of interstitial matrix, and cellular death by apoptosis and necrosis. HF is progressive, though the velocity of its progression varies depending on factors ranging from underlying cause to ethnical factors (93;141).

It seems very likely that the key to the progressive nature of LVH and HF is the activated signaling peptide systems. Several of these peptides have been shown to induce LVH when infused into animals, and their inhibition has succeeded in improving the otherwise very poor prognosis of HF patients. The most important of these signaling systems include the RA system, the

endothelin system, inflammatory cytokines and the kinin system, as well as the adrenergic system (93).

The balance between cardioprotective elements and components that are deleterious to the myocardium (cardiotoxic) determines the progression of heart failure and its velocity.

1.4 Cardiotoxic Pathways

Cardiotoxic pathways can be defined as signaling pathways that have deleterious effects on the myocardium and the cardiovascular system. Though the presence of these pathways is essential for normal cardiovascular physiology, the transition point from protection to toxicity is define.

1.4.1 Renin-Angiotensin System

The renin-angiotensin system (RAS) plays a prominent role in cardiac remodeling during HF development. Activation of the RA system induces formation of angiotensin II, which promotes myocyte hypertrophy, fibrosis, and chamber dilation in the heart, exerting similar effects to the β-adrenergic system. It also promotes water and sodium retention and exerts electrolytic disturbances (93).

The RAS involves systemic and local components. Although all the components of RAS are present in the heart, not all of them are believed to be synthesized there (341;494). The local RAS seems to be activated differentially depending on the activating signal and regulated independently from the circulating RAS (180;490).

Both local and systemic RA cascades are activated early during the progression of HF, and they are considered the major contributor to most cardiovascular diseases. In fact, the most important drugs for HF are based on blocking the RAS with ACE inhibitors, aldosterone inhibitors, or AT receptor inhibitors. Angiotensin converting enzyme (ACE), which converts Ang I into Ang II, also degrades bradykinin into its metabolites.

1.4.2 Endothelins

Endothelins are the most powerful vasoconstrictors identified. The effects of four different endothelin peptides (ET-1-4) are mediated through two G-protein-coupled receptors (ETA, ETB) that are distributed throughout tissues, notably the vascular endothelium, smooth muscle cells, and cardiomyocytes. In HF patients circulating ET-1 is elevated and correlates with the severity of hemodynamic disturbances and symptoms (300). Though endothelins are known to exert different effects on myocardial contractility in both normal and

failing hearts (272;442), vasoconstriction is the most apparent. However, the endothelin system is not as important as the adrenergic system and RAS for regulation of myocardial performance (312).

1.4.3 Cytokines

There is convincing evidence suggesting that inflammation plays a significant role in the pathogenesis of heart failure. Cytokines are highly potent endogenous peptides produced by a variety of cell types and act as essential mediators and modulators of many biological systems. Tumor necrosis factor (TNF)-α, interleukin (IL)-1α, IL-1β, and IL-6 have been classified as proinflammatory cytokines that play important roles in primary host responses and tissue repair. In experimental models, inflammatory cytokines promote left ventricular remodeling and acute reversible contractile dysfunction (299;429). In patients with chronic heart failure, increased plasma levels of proinflammatory cytokines, including TNF-α and IL-6, correlate with both the severity of disease symptoms and clinical outcome (256;456).

The cytokines act in concert with certain cytokine inhibitors and soluble cytokine receptors to regulate the human immune response. Cytokines such as TNF-α have an immediate negative inotrophic effect on the myocardium. Some of them are partly responsible for the increased oxygen consumption related to the impaired economy of contraction (189). Some interferons (IFN-γ, IFN-α) also depress the contractility of the myocardium and are involved in the progression of LVH and HF (88;422).

1.4.4 Adrenergic System

One of the most powerful deleterious systems is the symphatic or adrenergic system. The effects of adrenergic system include activation of α- and β-adrenergic receptors, which are targets of endogenous adrenaline or noradrenaline as well as exogenously-administered β-blockers (243). In chronic HF, increased adrenergic signaling is present and in continuous stimulation, a down-regulation especially of the β-1 adrenergic receptors is present (58). Desensitization of these receptors together with the reduced number of receptors, seen in HF, can be seen as a protective mechanism in the failing myocardium (58).

The adrenergic pathway is activated early in the course of HF and at first serves to compensate for the functional impairment of the myocardium, but ultimately results in deterioration of cardiac function, altered structure, and heart failure. Activation of the adrenergic system induces peripheral vasoconstriction, which in turn increases after-load and oxygen consumption. It causes hypoperfusion and increased anaerobic metabolism in tissues.

Excess noradrenaline increases intracellular calcium, which is directly lethal to cardiomyocytes. Sympathetic activation with excess noradrenalin stimulation promotes eccentric myocyte hypertrophy, enhances apoptosis of cardiomyocytes, and produces detrimental changes in contractile and metabolic proteins via alterations of gene expression in cardiomyocytes (90;135). Activation of the sympathetic system also promotes fibrosis and activates numerous other signaling systems such as the renin-angiotensin (RA) system.

1.5 Cardioprotective Pathways

When cardiotoxic pathways are activated in the myocardium, also opposing systems that protect the myocardium from deleterious signals are activated. To these protecting systems include, for example, the kinin, natriuretic, and adrenomedullin systems as well as the newly discovered urocortin and adiponectin systems. The kinin system is the main focus of this review and it will be closely examined later.

1.5.1 Natriuretic Pathway

In the late 1970s it was discovered that infusing atrial extracts into hypertensive rats lowered blood pressure and resulted in natriuresis (101). The peptide was later isolated and named atrial natriuretic peptide (ANP). Natriuretic peptides are a peptide family constituting ANP, brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP). ANP and BNP are mostly synthesized in the myocardium and secreted in response to muscle stress. CNP is considered a neurotransmitter in the central nervous system, but is also widely distributed throughout the vasculature and is found in high concentrations in the endothelium where it plays a role in the regulation of local vascular tone (495;506). ANP and BNP are widely used as indicators of myocardial ischemia and heart failure, where they can be used as prognostic biomarkers (320). The effects of natriuretic peptides are mediated through three receptors, namely NPR-A, NPR-B, and NPR-C. ANP and BNP bind preferentially to NPR-A and CNP prefers NPR-B. All three peptides bind to NPR-C, which seems to clear the natriuretic peptides from the circulation.

The peptides induce diuresis and natriuresis and also regulate blood pressure mainly via vasodilation by inhibiting the synthesis and secretion of aldosterone and renin. They inhibit vascular SMC proliferation and hypertrophy of cardiac myocytes and regulate systemic vascular permeability (203). Peptides act as circulating hormones, but also as autocrine and paracrine factors. In heart failure, the levels of ANP and BNP in plasma are increased and they are considered to compensate for heart failure (267).

1.5.2 Adrenomedullin

Adrenomedullin is a potent vasodilating peptide that was originally isolated from pheochromocytoma tissue (227). It is secreted by many cardiovascular tissues and cells including the myocardium, vascular endothelium and vascular smooth muscle cells (191). Adrenomedullin has similar effects as natriuretic peptides thus, it promotes vasodilation, natriuresis, diuresis, angiogenesis, antifibrotic effects, and anti-oxidative actions (227;324). Plasma adrenomedullin levels are increased in patients with various cardiovascular diseases, including hypertension, heart failure, and renal failure. Adrenomedullin has also showed to have an impact on the cardiac contractility and depending on the down-stream signaling cascades, it either decreases or increases the contractility of cardiomyocytes (57;253;430). Adrenomedullin as well as natriuretic peptides are mainly degraded in the human heart by neural endopeptidase (NEP).

1.5.3. Erythropoietin (EPO)

In response to ischemia, the cells upregulate erythropoietin through increased expression of hypoxia-inducible factor 1 α (HIF-1 α). Patients, suffering from chronic heart failure, are often anemic and recombinant-erythropoietin (EPO) has been successfully used in the treatment of HF patients (414).

Increased Hb in HF patients improves cardiac function, but EPO seems to exert also some non-hematopoietic effects. Erythropoietin has shown to have anti-apoptotic and antioxidative effects as well as angiogenesis promoting effects (373;418).

Recent studies showed that EPO treatment reduced the infarct size (both brain and heart infarction) in ischemia/reperfusion experiments. *In vivo* and *in vitro* studies reported reduced apoptosis in myocardial cells and normalized hemodynamic functions after reperfusion in rodents. Although the mechanisms of EPO in preserving cardiac functions are mainly unknown, the anti-apoptotic role of EPO has been speculated (69;463).

1.5.4 Other Cardioprotective Proteins

Urocortins (UCN) represent a recently discovered family of cardiac peptides. The name applies to three peptides (urocortin I-III) belonging to the corticotrophin-releasing-factor (CRF) super family. Three urocortins have so far been identified, and they exert their effects through two G-protein coupled receptors (269). Urocortin-I has shown to have potent coronary vasodilator and cardiac inotrophic effects. The effects resemble the effects of natriuretic peptides, and in animal models Urocortin I stimulates ANP and BNP secretion from cardiomyocytes (113).

Adiponectin is a protein secreted by adipocytes that has also been

connected to cardioprotective systems (340). It exhibits favorable effects on atherogenesis, endothelial function, and vascular remodeling by modulating the signaling cascades in vascular cells. Recent findings have shown that it can directly affect the signaling of cardiac cells and is beneficial in cardiac remodeling and also in acute cardiac injuries such as myocardial infarction (190).

Cardiotrophin-1 (CT-1) is a cytokine, also shown to have cardioprotective effects. The plasma level of CT-1 is increased in HF and it seems to have diagnostic and prognostic value in heart failure. The level of CT-1 is increased in hypertension, valvular diseases, hypoxia, and myocardial infarction and it stimulates cardiomyocyte proliferation. CT-1 has also shown to exert protective effects against ischemia/reperfusion injury, infused both prior to ischemia and at reperfusion. The results suggest that CT-1 has a protective role in myocardial diseases and it may play a role in decreasing myocardial damage (66;258).

On the basis of these findings, it has been hypothesized that a balance exists between harmful signaling systems and cardioprotective systems, which stabilizes the homeostasis of the normal heart. This equilibrium is disturbed in the beginning of the pathogenesis of HF, resulting in the onset of LVH, accompanied by remodeling and followed by impaired function of the heart, finally leading to heart failure.

2. The Kininogen-Kallikrein-Kinin Pathway

2.1 History

Kininogens are defined as circulating proteins that contain BK or lys-BK. These peptides and their active metabolites assume particular importance as natural ligands of two BK receptors (BK-1R and BK-2R). Interest in the kinin system originates from experiments done a hundred years ago by two surgeons who observed that an alcohol-insoluble fraction of human urine caused hypotension when injected into dogs (480). In 1937 Dr Werle discovered that kallikrein is a proteolytic enzyme that liberates the active polypeptide, kallidin, from the plasma protein kininogen (480). He also noticed the degradation of kinins and identified them as peptides (481).

In the late 1940s Rocha e Silva discovered that trypsin, when incubated with blood, released a substance that contracted the ileum of a guinea pig, and the substance was named bradykinin. He later succeeded in purifying the substance and determined that it was a peptide (378).

2.2 Kininogens

Kininogens are mainly synthesized by hepatocytes, and after posttranslational modifications they are secreted into the circulation (433). Two forms of kininogens have been identified in humans, namely high-molecular-weight kininogen (HMWK) and low-molecular-weight kininogen (LMWK), which differ in size, structure, and function. Their common basic structure – an aminoterminal heavy chain – is linked to varying carboxyterminal light chains, allowing the distinction between low- and high- molecular-weight kininogens (42).

BK and kallidin are formed from either HMWK or LMWK, respectively, by means of the enzymatic action of kallikreins. Several studies have shown that, at least, endothelial cells, platelets and granulocytes have binding sites for HMWK (168;399;465).

The binding of kininogens requires the presence of Zn^{2+} cations, suggesting it is necessary for the expression of the kininogen receptor (510). The affinity of LMWK and HMWK for binding is similar, but the number of binding sites for kininogens varies greatly between cells. Thus, platelets have approximately 1000 binding sites/cell and endothelial cells have between one and ten million binding sites/cell (92). When HMWK is bound to an endothelial cell it activates prekallikrein to release kallikrein, which in turn cleaves BK from HMWK (318).

The kininogen system is involved in inflammation and blood coagulation. In tissue destruction or thrombus formation the contact activation and coagulation activation occur *in vivo*, but the physiological function of the kallikrein system in coagulation has been challenged.

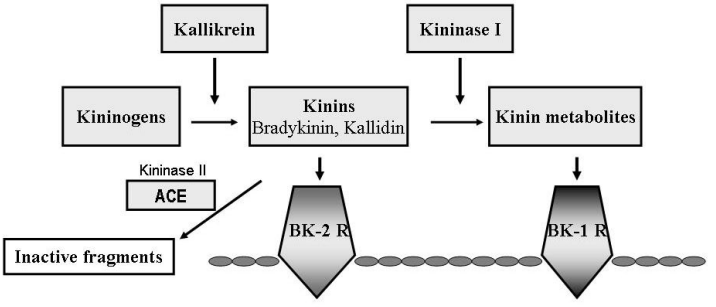

Fig 1. The human kininogen-kallikrein-kinin -system.

2.3 Kallikreins

Kallikreins are serine proteases found in glandular cells, neutrophils, and biological fluids. Kallikreins are divided into two groups: plasma and tissue

kallikreins, which differ in their molecular weight, pI (isoelectric point), substrate specificity, immunological characteristics, type of kinin released, and functional importance. Kallikreins release vasoactive kinin peptides through enzymatic action (321). Enzymes that possess the capacity to release kinins from kininogens are collectively called kininogenases, which include enzymes such as kallikreins, trypsin, neutrophil elastase, and plasmin (236).

Plasma prekallikrein (PPK) is synthesized and secreted by hepatocytes, forms a complex with HMWK, and circulates in the plasma attached to the external surface membrane of neutrophils (182). When the Hageman factor is activated, either by interaction with neutrophil or endothelial membranes or by binding to negatively charged surfaces, it enzymatically converts plasma prekallikrein to plasma kallikrein. A feedback mechanism causes further and rapid activation of the Hageman factor and together with cyclic conversion of PPK to kallikrein, results in local formation of kinins from kallikreins (37).

The major function of plasma kallikrein is to release BK from HMWK by hydrolysis of Lys-Arg and Arg-Ser bonds (217). It also participates in surface-dependent activation of blood clotting, fibrinolysis, regulation of vascular tone, and inflammation. Molecular events in blood clotting involve a number of proenzymes, namely plasma prekallikrein, factor XII (Hageman factor), factor XI, and HMWK, of which HMWK is essential for the coagulation cascade (217). Although LMWK is a poor substrate for plasma kallikrein, it can form BK in the presence of neutrophil elastase (387).

Tissue kallikrein (TK) is a single-chain acidic glycoprotein that differs completely from plasma kallikrein (37). Tissue kallikrein has strong kininase activity, and it is locally synthesized and releases mainly kallidin from both kininogens, though the main substrate is considered to be LMWK (184;273). Tissue kallikrein can also cleave other precursors to release active peptides (37). Tissue kallikrein has been isolated from various tissues, including the heart and arteries (34;302). Unlike with plasma kallikrein, continuous synthesis and secretion of kallikrein can occur in synthesizing tissues, producing kallidin from local and plasma-derived LMWK. This also means that as tissue kallikrein produces kinins continuously, it has an important role in the endothelial function in arteries. It also participates in blood pressure regulation and cardioprotection in early and late ischemic preconditioning (357).

Kallikreins can also activate BK-2Rs independently of kinin generation. This introduces a “shunt” concept into the kallikrein-kinin system in which the normal multistep-cascade to release bradykinin is effectively bypassed as kallikrein directly activates BK-2 receptors (39).

2.4 Bradykinin and Kallidin

Kallidin	Lys_1-Arg_2-Pro_3-Pro_4-Gly_5-Phe_6-Ser_7-Pro_8-Phe_9-Arg_{10}
Bradykinin	Arg_1-Pro_2-Pro_3-Gly_4-Phe_5-Ser_6-Pro_7-Phe_8-Arg_9

Fig 2. The structure of kinins

The two peptides usually referred to as kinins are bradykinin (BK) and kallidin (lys-BK). Their amino acid sequences are shown in **Fig 2**.

In human heart kallidin is rapidly cleaved into BK, and BK is rapidly metabolized into BK-(1-8), BK-(1-7), and BK-(1-5), shown in **Fig 3** (234).

Of the kinin metabolites, des-Arg^9-BK and des-Arg^{10}-KD can activate BK-1 receptors, and BK-2 receptors can be activated by both BK and KD. Of the bradykinin degradation products, BK-(1-7) is considered to be inactive and BK-(1-5) to be active in the coagulation cascade (177).

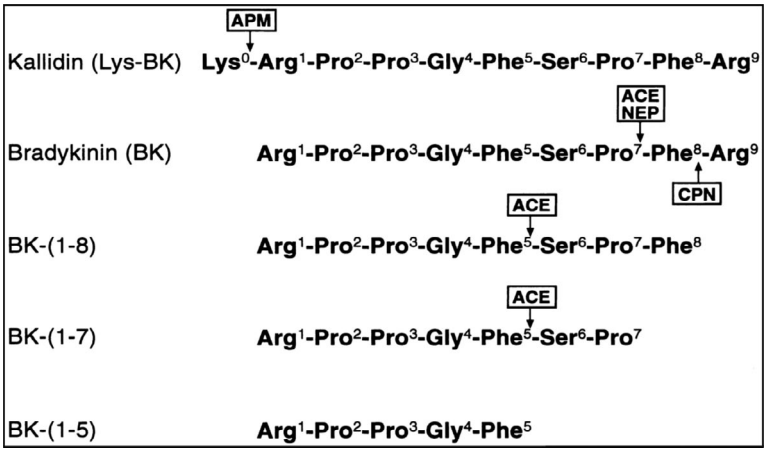

Fig 3. Kinins, their degradation products, and the degrading enzymes in human heart

In 1960 the first amino acid sequence proposed as bradykinin lacked a single proline residue in position 7; the correct sequence was established later that year (50).

The half-life of kinins is very short and they are rapidly degraded into inactive metabolites. In plasma the half-lives of BK and KD are estimated to be < 30 s (144;294). The concentration of BK in the circulation and tissues is very small, and the concentration of kallidin is even smaller (50). The concentrations (25-50 pM) of kinins in tissues are higher than in the circulation, reflecting the fact that tissues produce kinins and also that BK does not participate in systemic blood pressure regulation (314). Bradykinin

can be found in all secretions of the body, i.e. urine, saliva, sweat, and lacrimal fluid, as well as in tissues such as the heart, vasculature, blood, kidney, gastro-intestine track, lungs, skin, brain, and nervous system (72;302;403).

The most important hemodynamic effect of bradykinin is vasodilation, and vascular endothelial cells in the heart are the primary source of BK. Endogenous BK participates in the regulation of coronary vascular tone through BK-2Rs, thus vasodilation can be abolished by a BK-2R antagonist Icatibant. Exogenously-administered BK is also a potent coronary artery vasodilator (84;172;195;348). Vasodilation is partly achieved by stimulating vascular endothelial cells to produce NO but also by activating ECs to produce prostacyclin (PGI_2). The endothelium- derived hyperpolarizing factor (EDHF) is produced by activating BK-2Rs (172;226).

BK-induced vasodilation increases local hyperemia and reduces blood pressure, thus reducing systemic and local vascular resistance (172;397). It also stimulates nociceptive afferent nerves, promotes formation of edema by increasing vascular permeability, and participates in coagulation and fibrinolysis. BK induces antiproliferative effects via BK-2Rs by inhibiting several growth factors that inhibit the hypertrophy of myocytes, thus indicating that an intact kinin system is essential for the regulation of the myocardium (207;293;381).

Kallidin can be detected in the heart and circulation as well as in urine and the kidneys (127). Kallidin concentrations are lower than BK in both plasma (< 1 pM) and tissues (0.5- 4.0 pM), suggesting that the formed kallidin is rapidly degraded into BK (70).

Kinin concentrations are regulated by their formation, but especially by their degradation. There are many enzymes responsible for degradation of kinins, which differ from tissue to tissue, thus kinin profiles vary greatly between tissues. Also both BK-1 and BK-2 receptors, their secondary messenger pathways, and their responses to ligands differ, depending on the cell type and tissue observed. So, the same stimulation can cause entirely different activation in another tissue with the same ligand.

2.5 Kinin-degrading Enzymes

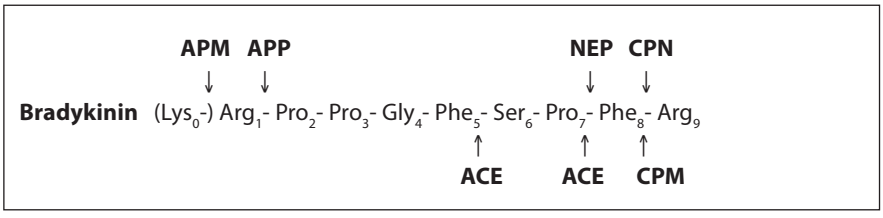

Fig 4. Sites and enzymes which degrade BK

Kinin-degrading enzymes are collectively called kininases. There are four major kininases that cleave kinins from the carboxyterminal end. There are also enzymes that cleave kinins from the aminoterminal end, but their significance seems to be less important – at least in the heart.

In humans, plasma BK is mainly metabolized by three metallopeptidases – ACE, APP, and CPN (41;99). In addition to ACE 1 a homologue of ACE 1 -ACE2-has recently been presented. In this review, ACE refers to ACE 1.

2.5.1 Aminoterminal-end-cleaving Enzymes

Aminopeptidase M (APM) is an enzyme detected in vascular endothelial and epithelial cells in the intestine and renal tubules, and it degrades kallidin into BK **(Fig 4)** (233;337;488). The concentration of kallidin in the human heart is very low, and kallidin is rapidly degraded into BK through the action of APM, likely by fibroblasts (233).

Aminopeptidase P (APP) is a membrane-bound enzyme located in endothelial cells, and particularly high activities have been located in the kidney, heart, and spleen. APP cleaves the first amino acid of BK, converting it into inactive BK-(2-9) (81).

In rats, APP together with ACE are the predominant kininases that participate equally in kinin degradation (108;137). A recent study with rats showed that inhibition of APP potentiated the cardioprotective actions of kinins (489). The importance of APP in humans emerges in the presence of ACE inhibition, when APP represents the main inactivating pathway of BK (99).

2.5.2 Carboxyterminal-end-cleaving Enzymes

The four major enzymes responsible for cleaving kinins from the carboxyterminal end are: angiotensin-converting enzyme 1 (ACE), carboxypeptidase's N and M (CPN, CPM), and neutral endopeptidase (NEP). Based on their enzymatic action, kininases are divided into kininase I and II. To the first belong CPN and CPM and to the latter, ACE and NEP. Kininase I cleaves carboxyterminal Arg-^{9} and kininase II cleaves terminal dipeptide Phe^{8}-Arg^{9} of kinins. **Fig 4** shows the main cleavage sites of BK.

ACE is mainly localized in the pulmonary vasculature, where it destroys more than 99% of the circulating BK in a single passage through the pulmonary circulation (367). It has generally been thought to be the most important BK-degrading enzyme in the heart and plasma (108;125). Accumulating evidence suggest that in human myocytes, BK is degraded mostly by NEP into inactive BK-(1-7), and only about 10% is degraded by ACE (126;233;354). The activity of kinins seems to be constant in humans in spite of cardiovascular diseases (233). In animals there is some evidence that the level of degrading enzymes

differs in diseased hearts (367). The degradation of kinins in the human heart is due to ACE, NEP, and APM, of which ACE is probably the most important in the endothelium, NEP in the myocardium, and APM in fibroblasts.

Kinins are degraded in plasma mainly by ACE, which cleaves bradykinin into inactive BK-(1-7) and into BK-(1-5), which accompanies the coagulation process.

3. Kinin Receptors

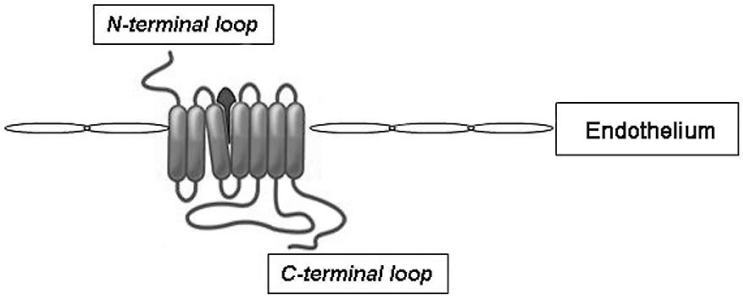

Fig 5. Schematic diagram of a G-protein receptor

Two types of 7-transmembrane G-protein-coupled receptors mediate the cellular effects of kinins, namely BK-1R and BK-2R. A third G-protein-coupled receptor named GPR-100 was recently detected and suspected to be a kinin receptor (49). Bradykinin binds to GPR-100 acts and it resembles BK-2R with a sequence homology of 27%. The receptor has now been confirmed as an insulin-like receptor with relaxin-3 (insulin-like peptide), a ligand (154;265).

The existence of BK receptors was suspected in the early 1970s (100;474;479). The first definition of the receptor was made in 1974 (29). In 1977 two different BK receptors were described in rabbit and rat aortas(369). Later the existence of the two receptors was confirmed, one being constitutively expressed (BK-2R) and the other being inducible (BK-1R) (369). The first findings of BK receptors in the heart were described in the myocardial nerve endings of dogs (425), and almost 20 years later in rat cardiomyocytes (307).

These pharmacologically different receptor subtypes are found interspecies, which explains some of the discrepancies in previous studies done with various animals.

Kinin receptors have been under investigation now for more than 30 years. The scientific interest in kinins arouse after the recognition that kinins induce contraction of smooth muscle cells in many vessels, but at the same time

stimulate the production of endothelial vasodilators, thus inducing vasodilation and relating kinins to cardiovascular diseases (85;445). The known positive cardiovascular effects are mainly mediated via BK-2Rs. Substantially less information exists about the functionality and significance of BK-1Rs.

3.1 BK-2 Receptors

3.1.1 History

BK-2Rs were first discovered in cat interstitium, rat stomach and uterus, and rabbit aorta and jugular vein by a in 1975 (28;29;369). The receptor encoding for 366 amino acid residues was cloned from rat uterus SMCs in 1991 (295).

Subsequently, the human receptor was cloned from a fibroblast cell line by PCR and cDNA library screening (183), and the human gene was encoded (128;215). The gene consists of three exons and two introns located in chromosome 14, specifically in 14q32. It codes for a 391 bp amino acid protein with a molecular mass of 41 Da, which has about 80% homology with mouse and rat BK-2 receptors (215;271;359).

3.1.2 Distribution and signaling of BK-2 Receptors

The "native" kinins – BK and kallidin – generated by plasma or tissue kallikreins in all mammalian species have a high affinity for BK-2Rs. None of the fragments of BK retain a significant affinity for BK-2Rs.

The constitutively expressed BK-2Rs have been identified in most tissues and cells: endothelial cells (146), smooth muscle cells (vascular and non-vascular) (36;38;381), and fibroblasts (183). They have also been detected in mesengial cells (13;285), neurons (426), neutrophils (27), and tumor cells, such as adenocarcinoma, lymphoma and hepatoma (491).

BK-2R signaling in the vasculature leads to either vasoconstriction or vasodilation (85;381) and promotes antithrombotic effects (59;407). Depending on the cell type, activation induces proliferative or antiproliferative responses in SMCs and fibroblasts, respectively (160).

Kinin receptors undergo multiple post-translational modifications, including glycosylations and disulfide bridge (cystine) formation, in their extracellular domains. Most of these modifications, including phosphorylation, occur in a ligand-independent manner, although phosphorylation and possibly also acetylation appears to be regulated primarily through receptor activation (355;424).

Of the post-translational modifications in BK-2Rs, glycosylation does not appear to affect ligand-receptor interactions (502). BK-2Rs contain two

cysteine residues in the tail region, which can be modified by palmitoylation, which further modifies receptor coupling, internalization, resensitization, and intracellular trafficking (355;424). Agonist -induced reversible phosphorylation plays an important role in the desensitization of GPCRs, which may also affect endocytosis, recycling, trafficking, and coupling of the receptors.

BK-2 receptors are phosphorylated upon stimulation, leading to receptor desensitization (147;210). Repeated or continuous stimulation leads to internalization of the desensitized receptors. Upon stimulation the receptors are redistributed into plasmalemmal caveolae, and the receptor-caveolae-containing vesicles are thereafter endocytosed and taken into cells. The receptors are recycled back to plasma membranes after a refracting period (43;46)

3.1.3 Physiological Effects of BK-2Rs

In animals and humans the vasodilation under normal conditions is mediated by BK-2Rs (43;46) and thus BK-2Rs mediate hypotensive effects. In the heart, BK-2Rs appear to be antiarrythmic (262), reduce infarct size, and contribute to preconditioning of the heart against ischemia (61;89;166;504)

BK-2Rs also have an impact on angiogenesis. In animal models receptor activation leads to angiogenesis via several downstream signaling pathways, notably activating eNOS, COX-2, ACE, and VEGF (133;310;415;443).

In the kidneys, BK-2Rs are expressed in all nephron segments, and receptor activation increases renal blood flow, and induces diuresis and natriuresis with no change in the glomerular filtration rate (205;279). Animal studies have shown that BK-2R activation in nephrons promotes antifibrotic effects (389;395). BK-2R activation prevented tubulo-intestinal fibrosis but not perivascular or glomerular fibrosis in hypertensive rats (185). In hypertension it has been shown that the local kinin system plays a significant role in renal protection (11;503). The protective effect is augmented with ACE inhibitors and vasopeptidase (dual ACE+NEP) inhibitors (405). Some conflicting results on BK-2R renoprotection have been published with BK-2R knock-out mice (392).

BK-2Rs affect glucose metabolism by stimulating glucose uptake in the presence of insulin (206;411), and hyperglycemia upregulates BK-2Rs in SMCs (86). The role of BK-2Rs in the development of type-1 diabetic nephropathy has been suggested but is controversial (208). Icatibant reduced glomerular hyper filtration, a phenomenon that is typical in the early phase of diabetes, but another study didn't find any differences in the progression of diabetic nephropathy in BK-2R -antagonized rats (12;453).

Studies of diabetic rats show activation of BK-2Rs and contribution to diabetic nephropathy, where activation of the connective tissue factor,

collagen I, and the transforming growth factor-β (TGF-β) are involved (435). The results have been confirmed in BK-2R -/- diabetic mice in which the renoprotective effect was associated with increased renal expression of BK-1Rs and AT-2Rs (435;436).

In the gastro-intestinal tract, BK and BK-2Rs induce SMCs to either relax or contract (17;145). The same phenomenon is also seen in smooth muscle cells in the uterus, bladder, and bronchi (209;450). The activation of BK-2 receptors has also been linked to inflammation and pain (116;117).

In the respiratory tract, kinins induce broncoconstriction, and BK and BK-2Rs are associated with the pathogenesis of asthma (116;117).

A potentially important role of BK-2Rs in asthma was demonstrated by a clinical trial in patients where icatibant dose-dependently increased pulmonary function test values. The effect was considered anti-inflammatory rather than bronchodilatory (9).

3.1.4 Induction of BK-2 Receptors

The stimulation of BK-2Rs does not influence the formation of kinins, implicating that there is no feed-back regulation between kinins and BK-2Rs (71;281).

The native ligand of BK-2Rs is bradykinin, but the receptors can also be stimulated by kallidin, kallikreins, or cathepsin G and trypsin (39;179).

Substantially divergent binding affinities have been reported for BK-2Rs in various tissues and preparations. The receptor affinity also depends on receptor coupling, and the phosporylation state and extent of receptor occupation (43;251;377).

3.1.4.1 Inflammation

Inflammatory cytokines can up-regulate BK-2Rs. In a T24 transitional carcinoma cell line, interferon-γ (IFN -γ) stimulated BK-2R expression (270). Interleukin 1-β (IL1-β) and the tumor necrosis factor-α (TNF-α) induce BK-2Rs through a post-transcriptional mechanism by activating PKA and p38 MAP kinase pathways (173;270;401).

In human airway smooth muscle cells, BK induces the release of IL1-β via cAMP, IL- 8, prostaglandin E2 (PGE_2), COX-2, and IL-6 by a BK-2R dependent pathway (327;344;507). As BK is considered proinflammatory in lungs, BK-2 receptors are possibly involved in the pathogenesis of asthma (30;78).

It has been suggested that BK-2Rs play a role in acute inflammatory response in contrast to BK-1Rs, which account for chronic inflammation (327).

3.1.4.2 ACE

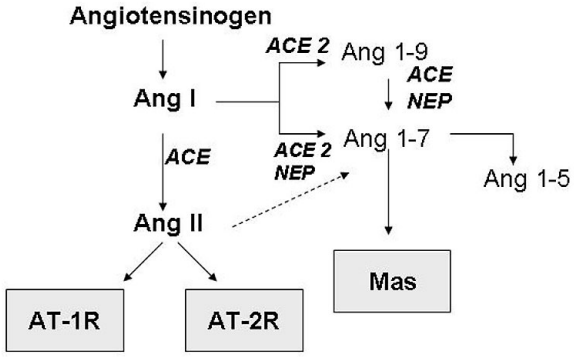

Fig. 6 The renin-angiotensin system. For abbreviations see the text below.

The renin -angiotensin system is a complex system that regulates the cardiovascular physiological processes. The primary effector molecule of this system is the potent vasopressor angiotensin II (Ang II), which has both beneficial and deleterious effects. Angiotensin-converting enzyme (ACE) is an ectoenzyme that generates Ang II by catalyzing its conversion from Ang I. In addition, ACE hydrolyses both bradykinin and kallidin (498). ACE preferentially hydrolyses bradykinin rather than Ang I and is especially abundant in the endothelial cells of lung vessels, but is also expressed in other endothelial cells, some smooth muscle cells, and monocytes (75). After a single passage through the pulmonary circulation, ACE degraded over 95% of administered BK in humans (51). ACE is heterogeneously distributed in the vascular tree, which may be of importance to regional blood flow (282).

ACE inhibitors exert beneficial effects on vascular endothelial function and vascular remodeling, many of which many are considered to be BK-2R-mediated effects (278;396). The beneficial effects are generally attributed to a decrease in Ang II and an accumulation of BK (278;483). ACE is also able to hydrolyse Ang II into Ang (1-7), which potentiates the vasodilating effects of BK, by inhibiting degradation but also by resensitizing BK-2Rs (142;446).

Ang (1-7) is a vasodilating peptide that counteracts the effects of Ang II. It acts by binding to a receptor of its own,- an orphan receptor,-Mas-,or to an AT-1 or AT-2 receptor, and participates in the regulation of cardiac function, coronary perfusion, and endothelial function (268;386). The vasoconstricting effects, described with high Ang (1-7) concentrations, are likely due to stimulation of AT-1R (358;446;459). Ang (1-7) is also formed by degradation of Ang I or II by a novel enzyme, -ACE-2 (115;471).

ACE-2 is a newly discovered enzyme that under normal conditions is located in the myocardium or vascular endothelium. It hydrolyzes Ang II into angiotensin (1-7), angiotensin I into angiotensin (1-9), cleaves kallidin but not BK, and is insensitive to ACE inhibitors (471). It has been suggested that ACE-2 counteracts the function of ACE. ACE-2 - KO mice have severely impaired cardiac function and thinning of the left ventricle wall, but not altered blood pressure or increased cardiac fibrosis. HIF-1-α related genes, induced by hypoxia (PAI-1 and BNIP3), are up-regulated, suggesting a role for ACE-2 in cardiac ischemia (96;115). There is also evidence, that ACE-2 is induced in the myocardium of heart failure patients (62;511).

Over the last decade there have been a number of reports showing that ACE and ACEIs amplify the responses of BK and BK-2Rs. Their cardioprotective effects are partly due to the reduction of BK degradation, but clearly there are other potentiating effects (193;483). ACEIs not only facilitate the accumulation of locally formed BK, they also directly affect BK-2R signaling, resulting in an enhanced response to BK. They stabilize BK-2Rs in a high affinity state, thereby preventing and/or reversing BK-induced sequestration to caveolae before internalization (33). The BK effects have also been suggested to be augmented by ACE-BK-2R cross -talk at the cell membrane (33;309). This could result from a steric relationship between the enzyme and the receptor or from heterodimer formation (283;308).

Both Ang (1-7) and Ang (1-9) induced reactivation of BK-2Rs in Chinese hamster ovary cells (CHO). The effect was attributed to a conformational change in the ACE-BK-2 receptor heterodimer (82;283).

All these results refer to a complex RAA-kinin system interaction, and despite the numerous studies on this subject, its physiological relevance is still not evident.

3.1.5 Inhibition of BK-2 Receptors

The first weak, non-receptor-specific antagonists of the BK-2Rs were generated in 1985 (467). A specific and potent antagonist HOE-140 or icatibant was introduced in 1991 (188) and is still the most commonly used antagonist, despite various chemical subclasses of BK-2R antagonists discovered later (14;383).

BK-2R inhibitors have been developed for clinical use and icatibant is currently used as a drug for treating angioedema. Unfortunately the results have not been as promising as hoped (31;175).

Stimulation of BK-2Rs with agonists reduces the affinity of the receptor towards ligands, thereby requiring higher ligand concentrations for stimulation (107). Long- term (> 24 h) stimulation of BK-2Rs reduces the surface binding sites by means of a post-translational mechanism that reduces receptor protein

synthesis and increase receptor instability (45).

The activation of BK-2Rs involves phosphorylation and desensitization of the receptor and subsequent internalization where the quantity of receptors decreases, which is also observed as the aminoterminal end of the receptor dimer (43;102). Depending on the ligand, recycling of the receptor can take a long time or it can be shortly restored to the plasma membrane. Internalization of BK-2Rs seems to be short-lived, since upon withdrawal of the agonists, a complete restoration of the receptor follows in 30 minutes (486). It also appears that BK-2Rs are almost completely recycled and therefore do not involve permanent down-regulation of the receptors (25).

BK-2R down-regulation is observed in some intense and chronic inflammatory states, but the mechanism is unknown (197). Nitric oxide can also inhibit BK-2R induction by inhibiting coupling with G-proteins (G_i,G_q) via a cGMP-dependent pathway (311).

3.1.6 Polymorphisms

Several polymorphisms of the BK-2R gene have been described. Point mutations occur in both the promoter region and the exons (56). Rieder's group recently sequenced 24 African-American and 23 Caucasian subjects and identified 77 SNPs of BK-2Rs. Twenty-five of them have a frequency greater than 10% and most of the discovered SNPs were located in the intron region. Two relatively highly frequent SNPs, with frequencies of 37%and 40%, were located at 102bp upstream and 277 bp downstream of exon 2. It has not yet been determined if these SNPs alter the function of the BK-2R gene and underlying physiological responses (374).

At least three single-nucleoside polymorphisms (SNP) of the BK-2R gene are known to have an impact on cardiovascular diseases. First, the promoter region -58T/C BK-2R polymorphism has been linked to hypertension in African-American, Chinese and Japanese populations. The C allele is an independent risk factor of essential hypertension (158;478). Fu *et al*. showed that the polymorphism is related to the development of LVH in hypertensive Japanese, but it didn't correlate with hypertension (155). Also the susceptibility to developing a cough with ACE inhibitors is associated with the -58T/C polymorphism (319). It is not clear if the T/ C transition causes an altered expression at the level of BK-2Rs.

Second, a point mutation at +181 C/T in exon 2 determines the Arg -> Cys substitution in the extracellular N-terminal domain. The potency of BK (EC_{50}) on BK-2Rs is selectively higher in the presence of the T allele. Furthermore, the T allele has been proposed to be protective during the development of renal failure (196).

And third, the deletion of 9 base pairs (-9/-9) in exon 1 seems to be the most

important polymorphism of the BK-2R gene. A lack of this deletion (+9/+9) is connected to a lower BK-2 receptor expression and is strongly associated with LVH growth response in normotensive men undergoing physical training (196). It also relates to impaired LV mass reduction during antihypertensive treatment (155;174).

3.1.7 Second Messengers of BK-2 Receptors

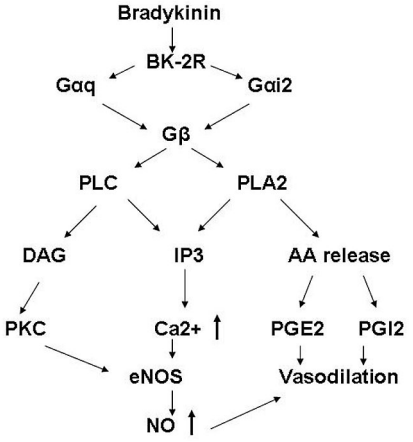

Fig 7. The intracellular signaling cascades of BK-2Rs
(For abbreviations see the text below)

BK receptors are seven trans-membrane G-protein-coupled receptors GPCR that, when activated transduce the provided information into intracellular second messengers. This process involves the coupling of activated GPCRs to various effector systems via their interaction with heterotrimeric G-proteins. Upon binding, the responses of the intracellular and also the intercellular mechanisms vary according to cell and tissue types as well as activators. The receptors form homo- and heterodimers and oligomers, which also modify their imminent responses. Another modifying factor is the initial stimulus, which can activate several different secondary pathways. The binding of a ligand activates G-proteins, namely $G\alpha_{i2}$ and $G\alpha_{q,}$ which activate phospholipases PLA_2, PLD, and/or PLC (257). The activation of the phospholipases induces the formation of inositol triphosphate (IP_3) and diacylglyserol (DAG). Upon stimulation, PLA_2 activates arachidonic acid (AA) release and prostaglandin (PGI_2 and PGE_2) production through COX-1 and COX-2 enzymes (160;496). The phospholipases induce an increase in

cytosolic Ca^{2+} and together with IP_3 and DAG they activate the protein kinase (PKC) pathway (43). The PKC pathway in turn activates various kinase and phosphate pathways. The increase of intracellular Ca^{2+} also activates eNOS to synthesize NO (63). eNOS is located in the plasma membrane near BK-2R and caveolin-1 complex. By BK-2R activation, eNOS dephosphorylates and initiates NO production (165;469).

The G-protein $G\alpha i_2$ is important in BK-2R signaling, thus the knockout model ($G\alpha i_{2\,-/-}$) displayed a two-fold increase in resting cAMP levels, which seems to be a BK-2R up-regulating factor, at least in fibroblasts (291).

In endothelial cells, BK-2Rs are located near the plasmalemmal caveolae (102). Caveolae are cholesterol- and glycosphingolipid-enriched invaginations of plasma membrane that function to bind, organize, and conserve a variety of signaling molecules (264). Several signaling proteins, including eNOS, interact with caveolin, an oligomeric integral membrane protein that appears to serve as the structural “scaffold” within caveolae (16).

When a ligand activates BK-2Rs, they normally transfer into the caveolae (102;211). The activated receptors are phosphorylated at Ser/Thr residues, located in the C-terminal domains and uncoupled from G-proteins (47;356). The receptors are internalized to an intracellular compartment by endocytosis and relocated in vesicles. By these actions the receptors are desensitized and can not respond to ligands. After internalization, dephosphorylated BK-2Rs can be resensitized and recycled back in to the plasma membrane (43). The cycle from receptor activation to resensitization and the capacity to bind a new ligand takes minutes, thus being a regulative element in BK-2R activation. The complex regulation of receptor sequestration and desensitization contributes to the differential use of the phosphorylation sites in BK-2Rs (46;356)

G-protein-coupled receptors (GPCRs) constitute a large family of proteins that converts external stimuli into intracellular activity. The receptors share a common deduced structure, and through their intracellular domains, they interact with heterotrimeric G-proteins. The activation of GPCRs initiates intracellular second messenger cascades, which ultimately evoke carefully controlled cellular responses (326).

The structures and functions of GPCRs are complex, and the secondary pathways vary from the cell to the participating ligand. Activation of BK-2Rs can induce vasodilatory effects on vessels via three different mechanisms. First, they can activate eNOS to produce NO, second they can activate $PLA_{2,}$ which promotes synthesis of prostacyclin. A third important vasodilating agent, the endothelial-derived hyperpolarizing factor (EDHF), is also related to BK-2Rs. The identity of EDHF is unknown, though a metabolite of arachidonic acid, epoxyeicosatrienoic acid (EET), is a strong candidate. EETs are synthesized the in vascular endothelium, they open calcium-activated potassium channels, and they hyperpolarize the membrane of vascular smooth muscle, conducing

relaxation (148). It has been proposed that EDHF consists of numerous endothelium-derived factors, including NO and PGI_2, but also reactive oxygen species, carbon monoxide, adenosine, peptides, and various metabolites of arachidonic acid, which can hyperpolarize the underlying smooth muscle thus initiating relaxation (139).

EDHF plays a significant role in resistant vessels, and it has been suggested as an important factor also in the vasodilation of coronary arteries, human forearm arteries, and renal arteries. It is thought to have relevance in preconditioning of the heart (410), and to contribute to vascular reactions in diabetes and other cardiovascular associated diseases (431;484).

The vasodilating substances NO, PGI_2, and EDHF partly cover each other, thus a lack of NO can be substituted by EDHF or PGI_2 (198;427). In eNOS knockout mice, EDHF completely replaces the lack of vasodilation normally produced by NO (427). Again, increased NO production inhibits the formation of EDHF, suggesting a physiological role for EDHF (224;363).

3.2 BK-1 Receptors

3.2.1 History

Regoli and co-workers also characterized BK-1Rs in the 1970s. The two G-protein receptors (BK-1R and BK-2R) were shown to differ in pharmacological profile and expression patterns (28;369;370). The gene was cloned from human embryonic lung fibroblast and is also located in chromosome 14, specifically 14q32(24). The receptor cDNA encodes the 353 amino acid protein with a molecular mass of approximately 41 kDa (303). The two human BK receptor genes are located consecutively in the same chromosome sharing a 36% genomic sequence homology. Mouse, rat, and rabbit BK-1Rs display functional and structural homology with human receptors, with rabbit receptors showing the greatest homology (24).

3.2.2 Distribution and Physiological Effects of BK-1 Receptors

In humans, BK-1Rs are weakly expressed under physiological conditions, but can be induced under pathological stimuli such as tissue injury or inflammation (67;369). However, constitutive expression of BK-1Rs is detected in the vascular compartment of rabbits, dogs, and cats (32;109).

BK-1 receptors can be detected in blood vessels, both veins and arteries, in cardiac tissue, kidneys, lungs, the central nervous system, ECs, SMCs and, fibroblasts (121;303;328). The distribution of BK-1Rs in vascular ECs and SMCs differs according to the size of the vessel: no BK-1Rs can be detected

in large veins or in the aorta (364).

Kinins able to induce BK-1Rs are des-Arg^{9}-BK and des-Arg^{10} -kallidin, bradykinin or other BK-2R inductors do not activate BK-1Rs (368).

The signaling of BK-1Rs is usually an endothelium-dependent phenomenon and can cause either vasoconstriction or vasodilation, depending on the species, the vessel, and its location (120;297). In most vessels the main effect is dilation, although in rabbit aorta and mesenteric vein as well as in human umbilical artery and vein, activation of BK-1Rs causes vasoconstriction (1;163;254;298;369;370).

Vasodilation is normally related to inflammation and is attributed to activation of eNOS and subsequently prolonged release of NO (109;120). The vasodilatory effects can be produced by prostanoids, mainly by PGI_2 and PGE_2, like in BK-2Rs (297;416). However, there are also conflicting studies where BK-1R-mediating vasorelaxation was not sensitive to cyclo-oxygenase inhibition, suggesting that prostaglandin release was not involved (362;385). This difference may be related to species and regional variation or to the absence of a specific prostanoid receptor subtype (297).

In some vessels, vasodilation mediated by the induction of BK-1Rs is endothelium-independent, which has been attributed to prostaglandin activity (376). BK-1Rs are positively coupled to phospholipase A_2 and induces arachidonic acid release (419). Despite prostaglandin synthesis, functional responses depend upon local PG sensitivity. Stimulation of BK-1Rs provokes PG synthesis and contraction in rabbit SMCs and bovine ECs, the contraction can not be prevented with indomethacin (121;254). PGI_2 and PGE_2 induce vasodilation in rabbit mesenteric and celiac arteries and rat coronary circulation, but fail to relax rabbit aorta, although BK-1R stimulation induces PGI_2 secretion (151;297;416) This indicates that not all endothelium-independent BK-1R-mediated vasodilatory effects are prostaglandin -mediated, despite the fact that prostaglandins are consistently released upon BK-1R stimulation. The prolonged response of activated BK-1Rs is probably related to the lack of desensitization and internalization of the receptors (22;289). Human BK-1Rs are not phosphorylated to any significant degree in either the absence or presence of ligands (44).

Many of the peripheral effects of BK-1R activation are pro-inflammatory: vasodilation, increased blood flow, increased plasma protein extravasation, and activation of leukocyte-EC interactions and leukocyte accumulation (8;296). Due to this, BK-1R studies have mainly focused on the regulation and expression of receptors in inflammation and infections (67;352).

The hypotensive responses mediated by BK-1R activation generally occur following inflammation and they have been extensively revealed (21;297;371).

Beyond the cardiovascular effects, BK-1Rs have been connected to diabetes

(512), chronic pain, and nociception (153) as well as cancer (365;438).

Although BK-1Rs and BK-2Rs seem to couple to similar signal transduction pathways, their signaling patterns differ. Also the impact on the differences in expression and regulation needs to be explored further.

3.2.3. Induction of BK-1 Receptors

Knowledge of the functionality and signaling mechanisms of BK-1Rs is incomplete. These receptors are present in tissues in negligible quantities and they can be induced by various inflammatory signals that produce vasoactive responses. BK-1R up-regulation occurs under the control of specific cytokines released in inflammation or after trauma (297;455). Unlike BK-2Rs, the stimulation of BK-1Rs does not desensitize receptors, indeed BK-2R desensitization can up-regulate BK-1Rs. This finding supports the hypothesis of an integrated system where BK-2Rs mediate acute inflammatory effects and BK-1Rs mediate sustained or chronic inflammatory responses. ACE inhibitors can also directly up-regulate BK-1Rs (202).

3.2.3.1 Inflammation

Induction of BK-1Rs is associated with production of inflammatory mediators, stimulation of inflammatory cells, and activation of several intracellular signaling pathways (351). Studies have shown that infection, with or without bacterial lipopolysaccarides (LPS), induces BK-1Rs and the vasodilation seen in septic infections is, at least partly, mediated by BK-1Rs (104;352).

LPS induces effects that are mediated through inflammatory molecules such as NFא-β, IL-1β, TNF-α, and PAF, which in turn promote the BK-1R up-regulation (346). LPS up-regulates BK-1R expression in rat coronary circulation, also possibly via direct action within the vessel wall (382).

Evidence from human lung fibroblasts suggests that receptor up-regulation involves activation of PKC, MAPK, and NF-kβ pathways (391;508). IL-1β is the optimal inducer of BK-1Rs in various cell types, and up-regulation modulates the receptors at both the transcriptional and post-transcriptional levels (391;508).

Activation of BK-1Rs reduces renal plasma flow and the glomerular filtration rate (GFR) in rats. This is attenuated when LPS is introduced prior to receptor induction, suggesting that activation of BK-1 receptors may participate in the modification of renal hemodynamics in inflammatory states (394). Over-expression of BK-1Rs renders transgenic-mice more susceptible to endotoxic shock and also exacerbates induced paw edema. The hemodynamic response to intravenously administered BK-1R agonist surprisingly increased mean arterial blood pressure in transgenic mice (329).

These results support a role for BK-1Rs in the modulation of inflammatory responses and hypertensive responses.

3.2.3.2 Allergy and Pain

Huan *et al* showed that allergen-stimulated bronchial hyper-reactivity in rats increased BK-1R expression, which was negated by blocking the receptor (199). Completely opposite results were published a few years later (130). The majority of animal studies support a role for BK-1Rs in lung inflammation, although the results have not been confirmed in human studies (136;372).

There is compelling evidence indicating that BK-1Rs play a key role in persistent inflammatory pain (67). Recent studies suggest a role for BK-1Rs also in neuropathic pain, which can be addressed as chronic and is in general very resistant to traditional medications. In transgenic mice, expressing human BK-1Rs, promising results were demonstrated when treating opioid -resistant, neuropathic pain with a highly human-specific BK-1R antagonist, (NVP-SAA164) (153). Another substance, namely ELN441958, has shown promising results in diminishing carrageenan-induced hyperalgesia in rhesus monkeys (178). Streptozotocin (STZ) -treated mice show characteristics of hyperalgesia, which can be completely blocked by a BK-1R antagonist (157).

3.2.3.3 Diabetes

In several animal studies, BK -1R up-regulation has been linked to diabetes (73;470). Streptozotocin-treated (STZ) diabetic rats showed long-lasting up-regulation of functional BK-1Rs in peripheral tissues, which could be negated with insulin treatment (73;94). A BK-1R antagonist (Leu^8-des-Arg^9-BK) was able to prevent insulin-dependent diabetes, presumably via an anti-inflammatory effect, in STZ -treated mice, but a BK-2R antagonist (Icatibant) was not able to do so (513). There are also some data concerning the development of diabetes that suggest that during the progression of diabetes, BK-1Rs are over-expressed and BK-2Rs are down-regulated (68).

A role for BK-1Rs has been suggested in the development of diabetes-associated retinopathies and neuropathies. A clear increase in BK-1R-specific binding sites is associated with an enhancement of des-Arg^9 -BK induced vasodilation in the retinas of STZ-treated rats (2).

3.2.3.4 Other Inducing Factors

Many studies suggest that ACE inhibitors can also directly activate BK-1Rs. Opposite results have been reported suggesting that ACE inhibitor-

induced BK-1R up-regulation might be an indirect effect, resulting from low endogenous kinin generation (201).

Increased NO production detected with ACE inhibitor treatment has been suggested to relate to direct BK-1R stimulation (201). The induction of BK-1Rs requires Zn^{2+} binding, inducing elevated Ca^{2+} levels and subsequent activation of eNOS and NO production, which can be blunted by BK-1R antagonist Ca-EDTA (201).

There are some indirect data from human heart failures suggesting that some of the vasodilatory effects induced by ACE inhibitor treatment are mediated through BK-1Rs (487).

It has been suggested that BK-2Rs and BK1Rs can "cross-talk" thus potentiating or preventing each other. Persistent stimulation or prolonged desensitization of BK-2Rs results in BK-1 receptor up-regulation, suggesting that the receptors can co-operate. BK-2R agonists can also directly activate the NFκβ pathway, which in turn can activate BK-1Rs (353;390).

A heterologous BK-1-BK-2 receptor-complex has been presented. Human BK receptors were spontaneously heterodimerized and the complex was associated with proteolytic degradation of BK-2Rs, suggesting a regulatory mechanism that participates in the transduction of a signal from BK-2Rs to BK-1Rs (216). This formation of a receptor complex may indicate a cellular adaptation of kinin signaling in sustained inflammation.

3.2.4 Inhibition of BK-1 Receptors

There are very limited data, on the inhibitors that influence BK-1R signaling.

Glucocorticoids and protein synthesis inhibitors have been shown to suppress the up-regulation of BK-1Rs in the rabbit aorta (103).

The number of BK-1Rs is constantly up-regulated in rats exhibiting low glucocorticoid levels, and by providing glucocorticoids the receptor levels are dropped back to normal levels (65).

The tumor suppressor factor, p53, can transcriptionally activate or silence a number of target genes, and it has been shown to inhibit the BK-1R gene (499).

3.2.5 Second Messengers of BK-1 Receptors

On activation, BK-1Rs couple to G-proteins, preferentially to the pertussis toxin insensitive G_g, or $G_{i,}$ through which it mediates stimulation (22).

The two BK receptors have many similar signaling pathways that involves elevation of intracellular Ca^{2+}, arachidonic acid release, eicosanoid production, and eNOS activation together with NO production (255;457).

3.3 Knock-out Models

3.3.1 BK-2 Receptor Models

A BK-2R knockout (KO) mouse was first generated by Borkowski in 1995. The mouse failed to respond to BK stimulation, such as smooth muscle contraction or afferent nerve stimulation. These mice are phenotypically normal and fertile and they have normal or only slightly elevated blood pressure, though malignant hypertension occurs with excess dietary salt (10;52). They have several alterations contributing to cardiovascular function: enhanced effects on Ang II, distorted renal development with abnormal nephrons and decreased glomerular capillary surface, and decreased renin and COX-2 expression in the kidneys (76;393).

Emanueli studied the cardiac phenotype of BK-2R knockout mice and reported the development of LVH accompanied by only a moderate rise in blood pressure in these animals by 2 months of age. LVH progressed to fulminant heart failure in six months without an increase in blood pressure, suggesting that remodeling was the primary process unrelated to systemic hypertension (132).

Progressive degenerative and fibrotic changes were observed in the myocardium of -/- mice and similar but less prominent changes were detected in heterozygous BK-2R +/- mice. The knockout animals were also tachycardic, similar to rats with a prenatal blockade of BK-2Rs, suggesting that kinins are also involved in early programming of the cardiovascular phenotype (274). Increased myocardial weight and blood pressure at the base-line has been reported in knockout mice, though others have failed to repeat these results (11;275;448).

Duka *et al.* reported that BK-2R knockout-mice developed LVH and heart failure after myocardial infarction and that remodeling and cardiac dysfunction responded poorly to ACE inhibitor or ARB treatment. The same group later reported that ACE inhibition worsened the cardiac function in mice lacking BK-2Rs. This effect was linked to up-regulation of pro-inflammatory BK-1Rs (122;124).

In BK-2R knockout mice, BK-1Rs are up-regulated and their sensitivity to stimulation is significantly increased, which partially substitutes for the lack of BK-2Rs. Further, stimulation of BK-1Rs with an agonist provokes hypotension and antagonist-induced hypertension, suggesting an antihypertensive effect of BK-1Rs, possibly through inhibition of vasoconstrictors produced through the AA cascade (123;124).

Kakoki *et al.* reported that in the absence of BK-2Rs, Akita diabetic mice show an additive increase in oxidative stress, mitochondrial DNA damage, and other senescence-associated phenomena, implying that increased senescence

and apoptosis in actively dividing cells are related to BK-2Rs. The authors suggest that the increased oxidative stress and ROS are due to decreased intracellular NO production in BK-2R-deficient diabetic mice (212).

The transgenic mice models have further verified the role of BK-2Rs in hypertension. Over-expression of human BK-2Rs in mice induces hypotension, which can be negated by icatibant. Also rats that over-express human tissue kallikrein gene experience hypotension. Induced hypertension with subsequent LVH was significantly reduced in hTK over-expressing rats compared with controls, and icatibant completely inhibited the effects (413).

3.3.2 BK-1 Receptor Models

The BK-1R knockout mouse was generated five years later than the BK-2R KO (352). The lack of BK-1Rs has no effect on blood pressure or cardiac performance in physiological situations.

Induced myocardial infarction in BK-1R -/- mice did not significantly influence myocardial remodeling (493). In another study BK-1R knockout mice showed higher coronary perfusion pressure, indicating higher coronary resistance. eNOS expression was also significantly diminished in the aortas of -/- mice, which could explain their increased coronary resistance (249). BK-1R knockout-mice have a pain-phenotype that implicates a role for this receptor in inflammatory and neuropathic pain. Knockout-mice exhibit reduced responses compared to wild types, in several pain models (143;352).

The absence of BK-1Rs in LPS-induced septic shock is associated with significant hypotension and significantly increased mortality (297).

The relevance of BK-1Rs in LPS-mediated septic shock has been intensively studied in knockout-models and the results have been conflicting: some report hypotension and others hypertension in septic BK-1R knockout-mice (74;352). Experimental differences explain some of these discrepancies, and it has been suggested that the BK-1Rs are first up-regulated in the initial phase of septicemia and are associated with vasoconstriction, implying that the BK1-Rs protect against acute, significant hypotension, thus reducing mortality (406).

Reduced neutrophil migration to inflamed tissues as well as apoptosis of BK-1R expressing neutrophils as a requirement for the resolution of inflammation was reported in mice lacking BK-1Rs (352).

3.3.3 Double KO

Until recently, the close proximity of the genes encoding the two receptors has prevented the generation of double-KO mice. Surprisingly double- KO -mice are normotensive and their cardiovascular morphology is normal, supporting

previous results indicating that under basal conditions, the kinin system does not play a significant role in cardiovascular physiology, at least in mice (74).

Table I. The main (cardiovascular) effects of BK-Rs

	BK-1R	BK-2R	BK1R ref	BK-2R ref
Vasodilation	++ (artery)	++	(103;120;297)	(85;87)
Vasoconstriction	++ (vein)		(1;163;369)	
SMC effects	anti-proliferation	anti-proliferation	(112)	(381)
VSMC contraction	+	++	(109)	(254;255)
Ischemic preconditioning	+	++	(53;77)	(61;89;468;475)
Angiogenesis	+	+	(112;345)	(133;310;443)
Fibroblast	pro-fibrotic	anti-fibrotic	(181)	(389;395;454)
Inflammation	chronic	acute	(68;73;298)	(298)
Leukocyte trafficking			(35;296;352)	
Cytokine activation			(104;172)	(173;270)
Endotoxic shock	hypotension	early hypotension	(103;297;371)	(298)
Pain	chronic	acute	(351;352)	(67;117;351)
Thrombosis		anti-thrombotic		(59;361)
Bronchoconstriction	+	+	(199)	(37)

4. Smooth Muscle Cells

In addition to endothelial cells, the BK -receptors can also be detected in other cells, though it is believed that their major effects are contributed via ECs.

It is known that in smooth muscle cells, BK-2R stimulation causes vasoconstriction and contributes to vascular injuries. In denuded canine saphenous veins, direct induction of BK onto SMCs induced concentration-dependent vasoconstriction, which seemed to involve BK-2Rs and arachidonic acids, implying PGI_2 involvement (287).

In experiments with intact guinea pig aortic rings or arterial myocyte cultures, direct BK-2R stimulation leads to constriction.

When the vascular endothelium is damaged, stimulants can gain direct access to the receptors in the smooth muscle. In these circumstances it is possible that BK-Rs play a role in the regulation of vascular injury.

5. Angiogenesis

Angiogenesis is the process of forming new capillaries from existing blood vessels. It involves several steps that result in the formation of new vessels when the highly regulated balance is in favour of angiogenesis (149).

The pro-angiogenic effect of BK has been demonstrated in both *in vitro* and *in vivo* studies under different experimental settings (79;131;231;317;345). Some data on cardiac angiogenesis are also available, which show favourable effects of kinin receptors on angiogenesis. It was recently shown that angiotensin II induced angiogenesis in hypoxic mouse hearts in a BK-2R- and NO-dependent way that also involved AT-2Rs (322). Interestingly, neither Ang II nor VEGF could induce angiogenesis in BK-2R knockout-mice (322). BK-2Rs transactivated the VEGF receptor (KDR/Flk1) in cardiac capillary ECs, resulting in further eNOS activation (443). Later the results were confirmed with coronary endothelial cells, where BK induced tube formation through a BK-2R-KDR/Flk1-dependent mechanism. BK and VEGF were suggested to have a synenergistic effect on angiogenesis (310).

Angiogenesis has also been linked to BK-1Rs (345). The study showed that BK was able to activate BK-1Rs in rabbit corneal capillary endothelial cells by stimulating the eNOS/FGF-2-pathway. These results were confirmed later in a study that showed a BK-1R dependent up-regulation of eNOS, and FGF-2 was involved in microvascular coronary endothelial cell and migration was induced by ACE inhibitors (114).

Tissue kallikrein has been associated with angiogenesis in several transgenic studies.

Transduction of the human tissue kallikrein (hTK) gene into an ischemic myocardium stimulated arteriole and capillary growth, reduced apoptosis, and increased the number of cardiac progenitor cells. A transient increase in TK was detected and the Akt-eNOS-NO-pathways were activated through increased kinin production (133;176). Unfortunately, these studies did not explore the BK receptors responsible.

In another study, hTK gene delivery improved cardiac function and protected against cardiac remodeling in HF by attenuating cardiac hypertrophy, fibrosis, and LV enlargement and by promoting capillary formation. These effects were seemingly connected to kinin-BK-2R activation (6;48;505).

These studies suggest that BK modulates cell-signaling pathways through BK1-and/or BK2-receptors and thus plays a role in regulating angiogenesis. The specific mechanisms underlying angiogenesis and the significance of BK receptors remain under debate.

6. Endothelial Dysfunction

6.1 Endothelium General

The endothelium is a continuous monolayer of cells linked to each other by different type of adhesive structures or junctions that cover the cardiovascular system. For a long time the endothelium was thought to be an inert layer covering the vessels but today we know that it is a dynamic structure that regulates and maintains cardiovascular homeostasis (315).

The endothelium is only 0.5 μm thick and rests on a thin basement membrane. The single cells are connected to each other by junctions (241). During embryonic development, endothelial cells differentiate from common precursor cells and acquire organ-specific properties. An important determinant of the cell differentiation is the local environment, and especially interactions with surrounding cells. These interactions may occur through the release of soluble cytokines, cell-to-cell adhesion, and communication or synthesis of matrix proteins on which the endothelium adheres and grows. Even in the same organ, the endothelium exhibits significant heterogeneity (375).

Two types of endothelial cells are recognized in the heart: endocardial endothelial cells and microvascular endothelial (MVE) cells (241). Both cells produce the same autacoids and share a similar role in signal transduction. The huge difference between them is that the MVECs contribute to cardiac function indirectly by regulating regional blood flow, but the endocardial ECs function directly by communicating with myocardial cells.

The endothelium contributes to cardiovascular homeostasis by regulating vascular permeability and adjusting the caliber of blood vessels according to hemodynamic and hormonal demands. Endothelial cells perform these functions by expressing, activating, and releasing powerful vasoactive substances and other bioactive molecules. These substances include factors that affect vasoconstriction and -vasodilation, coagulation, thrombosis formation, growth, angiogenesis, and tissue remodeling as well as immune reactions and tissue inflammation. The endothelium thus creates a complex and finely tuned balance of interactions with the immediate environment, which are common to all endothelial cells (204;380).

Despite its stable constitutive properties, a normal endothelium reversibly changes its function on activation. For instance, exposure of ECs to inflammatory cytokines induces reprogramming of genes - activation of some and depression of others (280). Senescence of ECs influence endothelial responses, thus senescent cells have defective signaling pathways that lead to an inability to properly responds to stimulants such as growth factors (161).

6.2 Endothelium -derived Vasoactive Substances

Endothelial cells secrete vasodilating mediators in response to substances released from autonomic and sensory nerves, circulating hormones, coagulation derivates, or autacoids such as bradykinin, angiotensin, and endothelin, secreted by endothelial or smooth muscle cells. Some of these vasodilating substances possess angiogenic and growth-modulating properties. Inflammation, vascular lesions, and thrombosis often accompany impaired vasodilation. It is generally accepted that the net effect of noxious substances and vasoactive, retrieving substances dispose the condition of the endothelium. Thus, there are several mending mechanisms in the endothelium that oppose dysfunction -promoting stimuli, of which the most important ones are mentioned here.

Activation of the kinin receptors induces three of the important mechanisms by which the endothelium reacts by vasodilation, as shown in **Fig 8**.

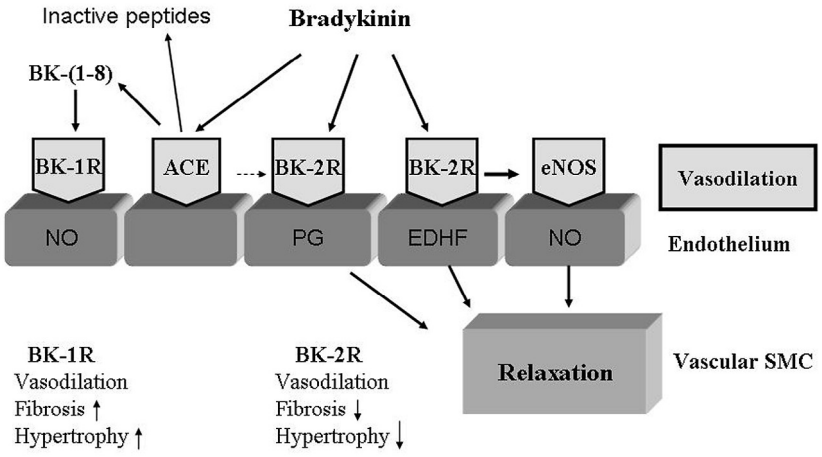

Fig 8. A schematic view of the mechanisms of BK-BK-2R activation in the endothelium

6.2.1 Nitric Oxide

Nitric oxide (NO) secreted by vascular endothelial cells is a ubiquitous signaling molecule that is able to diffuse across membranes, producing various physiological effects, of which the most important effect is vasodilation (200;343). Three different NOS isoforms have been described, named after the cell type first isolated. They differ in tissue expression, regulation and the average amount of NO produced. The endothelial (eNOS) and neuronal (nNOS) isoforms are constitutively expressed; iNOS is induced under pathological conditions and can produce vast amounts of NO. The synthesis

of NO requires L-arginine as a substrate and binding of Ca^{2+} / calmodulin. BK also stimulates NO production through Ca^{2+}/calmodulin-mediated eNOS activation.

The NO synthases are NADPH-dependent oxygenases that require several co-factors.

eNOS is the dominant enzyme in vascular cells and it is localized in the endothelial caveolae and negatively regulated by caveolin. Indeed eNOS seems to be mainly regulated by modulation of its activity, e.g. by protein kinases (MAP, Akt) (162;171). The inducible NO synthase (iNOS) is primarily regulated transcriptionally and is independent of agonist stimulation or intracellular calcium level (162;171).

A lack of NO is a predominant finding in endothelial dysfunction, which can be created by blocking eNOS and ameliorated by introducing nitrates. Activation of BK-2Rs, but also BK-1Rs, stimulates eNOS to produce NO, leading to vasodilation (132;493).

6.2.2 EDHF

The endothelial-derived hyperpolarizing factor (EDHF) contributes to vasodilation and involves BK-2Rs. Many molecules have been suggested as being the EDHF, and lately the notion has focused on arachidonic acid metabolites, mainly epoxyeicosatrienoic acids (EETs). The origin of the EDHF is still unclear (148). The effect of the EDHF has been demonstrated in various blood vessels from different species, including humans, and it likely plays an important role in cardiovascular physiology. In human coronary arteries, the importance of the EDHF seems to increase as the arterial diameter decreases (461). EDHF-mediated responses are clearly altered in various pathological conditions such as heart failure, atherosclerosis, and hypertension (140).

6.2.3 PGI_2

The vasodilating prostaglandin -prostacyclin (PGI_2)- is primarily produced by ECs (316). BK-2R stimulation activates PGI_2 release through the PLA_2 and arachidonic acid pathway (AA). However, unlike NO, the vasodilating effects of PGI_2 are determined by the expression of specific cyclo-oxygenases (COX-1 and COX-2) in vascular smooth muscle cells, and if the arteries do not express these binding receptors, prostacyclin can not participate in vasodilation (91). Receptor activation in SMCs elevates cyclic nucleotide levels, inducing hyperpolarization thus inhibiting vasocontraction. The link between NO and PGI_2 is bidirectional: prostacyclin can ease the release of NO from ECs and NO potentiates the effect of prostacyclin in vascular SMCs (106;409).

6.2.4 PGE_2

Prostaglandin E_2 is another prostaglandin participating in a wide range of cardiovascularfunctions, such as vasoconstriction and vasodilation, control of blood pressure, regulation of smooth muscle tone and modulation of inflammation. It has been connected to angiogenesis, though the mechanisms are not completely understood, thus PGE_2 does not stimulate EC migration (187;417), but can induce vascular endothelial growth factor (VEGF) expression and thus relate to angiogenesis via VEGF (417).

6.3 Endothelial Dysfunction

Endothelial dysfunction refers to a pathophysiological state where there is an imbalance between the endothelium-derived relaxing and contracting factors and the availability of NO, in particular, is diminished (466).

This imbalance leads to reduced vasodilation and to activation of pro-inflammatory and pro-thrombotic processes, promoting endothelial dysfunction.

Endothelial dysfunction plays a central role in the initiation and progression of cardiovascular diseases in which the pathophysiological end point is alteration of vascular homeostasis, which leads to diminished tissue blood supply. Diseases like heart failure, coronary arterial disease, and hypertension are all associated with endothelial dysfunction, where diminished availability of NO and increased reactive oxygen species (ROS) formation is detected. EC dysfunction is also related to factors such as smoking, hyperglycemia, and hypercholesterolemia, which are all major risk factors of cardiovascular diseases, but are also connected to the kinin system. Despite the many provoking factors, by far the most important perpetrator of endothelial dysfunction is hypoxia.

6.3.1 Hypoxia

The origin of various vascular pathologies involves instant or recurrent oxygen deficiency. Hypoxia activates endothelial cells by means of a complex system that varies between species, but also between cell types, where the same hypoxic effect can induce different signaling. At first the activation can be viewed as adaptive or protective, but if the stimulus is severe or last long enough, the reaction changes into a maladaptive or lethal one. Following acute hypoxia, endothelial cell activation, neutrophil adherence with local inflammation, and enhanced production of cytokines (IL-6, IL-1β, MCP-1) are observed, accounting for the inflammatory response in ischemic tissues (412).

Hypoxia increases the cytosolic calcium concentration that regulates many enzymes and participates in signal transduction, such as phospholipase A_2

(PLA_2), which increases in hypoxic ECs, leading to prostaglandin synthesis (20;306). It also up-regulates COX-2 through the induction of NF-kappa-β expression, which also induces ECs to secrete prostaglandins (400).

Even though the endothelial cell layer is relatively tolerant of low oxygen tensions, many of the effects of chronic hypoxia are thought to take place through alterations of endothelial cell signaling. Hypoxia directly affects the release of vasoactive nitric oxide (NO), which locally regulates vascular smooth muscle tone (473), though the physiological mechanisms regulating NO production in the vasculature are not completely understood. Conflicting results on eNOS expression and regulation under hypoxia have been presented and the results seem to vary between species and organs (301;434).

Together with eNOS, also iNOS can be induced in endothelial cells, so the net effect of NO production is even more complicated (342).

In chronic hypoxia, the expression of growth factors, cytokines, and molecules participating in the coagulation process is augmented, affecting remodeling of the vascular wall and myocardium. Many mitogenic molecules released by ECs induce smooth muscle proliferation in vascular beds (338). By far the most potent mitogen in ECs is the vascular endothelial growth factor (VEGF), which initiates angiogenesis *in vivo*. Hypoxia up-regulates the VEGF, but also its receptor, which may explain the strong mitogenic response during hypoxia (476).

Hypoxia transcriptionally induces several genes. The major regulator in cells and tissues is the hypoxia-inducible factor (HIF), an oxygen-sensitive transcription activator. The complex of HIF consists of two proteins: hypoxia -induced HIF-1α and constitutively expressed HIF-1β. The activated complex is associated with a specific binding site in target genes and it binds transcriptional co -activators to induce gene expression (214). Today there are over a hundred HIF downstream genes identified, and more that 2% of all human genes are directly or indirectly regulated by HIF in arterial endothelial cells (277). HIF participates in multiple physiological responses to hypoxia, such as erythropoiesis, glycolysis, angiogenesis, and apoptosis.

Many genes, such as VEGF, involved in angiogenesis are activated in hypoxia and regulated by the HIF. The levels of HIF-1α and VEGF are up-regulated in the myocardium of patients with acute coronary occlusion (250). Effective vascular remodeling in ischemic injury depends on an integrated program of HIF-dependent gene expression (223). Although the HIF is activated in situations where also the kinin system is activated, there are no data relating these systems.

6.3.2 Other Factors Contributing to Endothelial Dysfunction

Hypercholesterolemia induces impairment of endothelium-dependent

vasodilation. Oxidized low-density lipoproteins (LDL) down-regulate the expression of eNOS protein and inhibit the release of both NO and the EDHF, leading to impaired vasodilation (129;472). By disturbing the balance in the endothelium, LDL promotes several vasoconstricting factors (259).

Hyperglycemia with or without insulin deficiency is a major contributor to endothelial dysfunction. Hyperglycemia and its biochemical sequelae directly affect the endothelial function, but also the synthesis of vasoactive substances, growth factors, and cytokines. It is not clear whether impaired endothelial function is due to hyperglycemia or other factors. It is also closely associated with the presence of insulin resistance, regardless of the presence of diabetes.

In hypertension there is an imbalance between the vasoconstrictory and vasodilatory-systems, in particular a reduced vascular availability of endothelium -derived NO. In SHR aorta the endothelium induces cyclo-oxygenase-related contraction of the underlying vascular smooth muscle cells in response to contracting factors. Patients with essential hypertension have impaired dilatory responses to BK and other stimulants, which can be partially restored by indomethacin (466). Impaired BK -dependent vasodilation in hypertension is also found in normotensive people with a family history of hypertension (432). Several other factors, such as renal insufficiency, aging, smoking, hyper-homocysteinemia, and estrogen-deficiency, also contribute to endothelial dysfunction (330).

6.3.3 Endothelial Dysfunction in Heart Failure

Endothelial dysfunction has been presented in cardiac failure in both animals and humans (222;440). It is found in HF patients with normal coronary circulation or no predisposing factors, suggesting that dysfunction could represent an independent factor in the development of HF (335;449). These patients have impaired relaxation of the coronary microvasculature and peripheral vasculature, but enhanced basal production of NO (237). The relaxation is heterogeneously affected in different vascular beds (335). The damped vasodilating reaction in HF patients together with the release of vasoconstricting substances facilitates poor perfusion and ischemia of the myocardium (118;335).

In animal models, the induction of LVH up-regulates the expression of eNOS.

However during the transition to HF, the expression of eNOS also reduces the secretion of NO (170). This regulation of eNOS has been shown in human end-stage HF and is consistent with BK-2R expression in the progression of HF (239;240).

7. Treatment

The treatment of cardiovascular diseases has progressed during the last decades. As knowledge about the diseases and the molecular mechanisms involved has increased, more possibilities have become available. Despite the increased knowledge and treatment of heart failure, the prognosis has not improved much.

The medical treatment of HF consists of diuretics and vasodilating drugs, such as nitroglycerin, which both relieve the symptoms but have no impact on the prognosis of HF. The β-blockers, previously believed to increase the mortality in HF, are now recommended for the treatment of HF together with diuretics and ACE inhibitors, and shown to have both symptom relieving but also mortality reducing effects also in HF (110). ACE inhibitors/AT-1 receptor inhibitors and statins are widely used in the treatment of HF and they will be discussed here more closely.

7.1 Statins

Statins are in many ways intriguing drugs that were originally developed to lower plasma cholesterol.

Cholesterol is an essential component of cell membranes and also a precursor of steroid hormones and bile acids. However, in excessive quantities, cholesterol becomes a major risk factor of cardiovascular diseases. The rate -limiting enzyme of cholesterol biosynthesis in the liver is HMG-CoA reductase, which catalyses the conversion of HMG-CoA into mevalonic acid (mevanolate) **(Fig 9.)**. Inhibitors of HMG-CoA reductase, statins were isolated in 1976 from *Penicillium citrinium*, later named as mevastatin (134). A few years later a more potent substance, lovastatin, was isolated and later approved for clinical use. Since then, several new potent statins have been developed and they are today in wide clinical use.

Statins are potent drugs that cause a dramatic reduction in circulating LDL-cholesterol and an increase in LDL clearance. However, increasing data show that the beneficial effects of statins are only partially cholesterol -dependent and possess many cholesterol-independent or pleoitropic effects.

The Framingham heart study demonstrated that elevated cholesterol is an important risk factor of cardiovascular diseases. The mechanisms attributed to lipid lowering with statins include modification of atherosclerosis progression, atheromatous plaque stabilization, and improved endothelial function. Hence, statins reduce cardiovascular events in both hypercholesterolemic and normocholesterolemic patients with coronary heart disease.

In several studies (4S, CARE, HOPE, LIPID, WOSCOPS) statins have demonstrated beneficial effects in both primary and secondary prevention

of cardiovascular diseases. They also improve heart function and survival in patients with non-ischemic heart failure (194;331). It has been suggested that short-term statin therapy improves cardiac function, neurohormonal imbalance, and symptoms associated with idiopathic dilating cardiomyopathy, suggesting that a non-cholesterol -dependent mechanism is involved (150;194;331).
On the other hand, there are also hypotheses, which compromise the beneficial effects of statins in HF, of which the lipoprotein-endotoxin -hypothesis is well known (366).

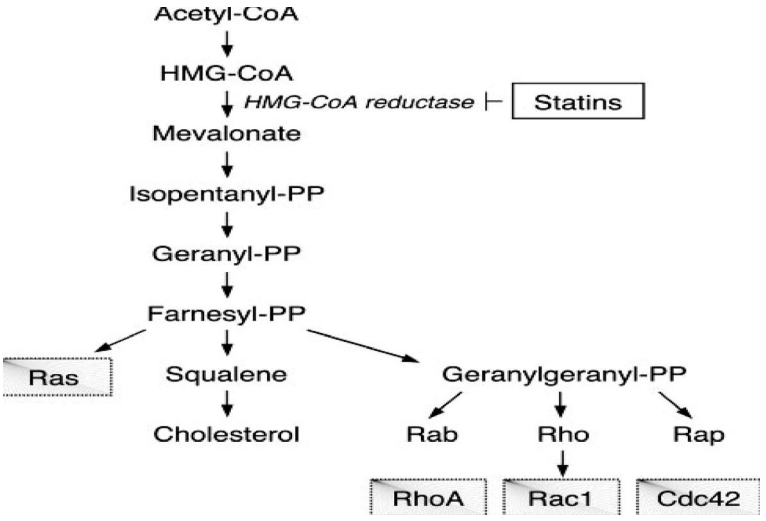

Fig 9. The cholesterol pathway

By inhibiting the conversion of HMG-CoA to mevanolate, statins prevent the synthesis of important isoprenoids such as farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP), which are precursors of cholesterol but also serve as important intermediate lipid attachments for translational modification of a variety of proteins including small guanosine triphosphate (GTP)-binding proteins Ras, Rho, Rac, and Rab (464). **(Fig. 9.)** Protein isoprenylation permits covalent attachment, subcellular localization, and intracellular trafficking of membrane-bound proteins. Members of the Ras and Rho-GTPase families are important substrates for prenylation, and statins inhibit this action, leading to accumulation of inactive Ras and Rho proteins in the cytoplasm (464). The Rho GTPase -family consists of RhoA, Rac, and Cdc 42 protein subfamilies. The overall functions of Rho GTPases are related to cell shape, motility, secretion, and proliferation. Thus, statins induce a change in the three-dimensional actin cytoskeleton, affect intracellular transport, membrane trafficking, and mRNA stability as well as gene transcription (246). The activation of Rho and its downstream target-

Rho-associated -protein kinase (ROCK) - increases the sensitivity of vascular smooth muscle cells to calcium in hypertension and coronary spasm (218;460). The activation is connected to smooth muscle contraction, oxidative stress, aortic stiffness, and changes in blood pressure. The pathway is also involved in myogenesis, angiogenesis, adipogenesis, cardiac hypertrophy, hypertension, and perivascular fibrosis as well as in the activation of inflammatory cytokines and chemokines (333;423;460).

The Rac pathway is connected to the formation of reactive oxygen species (ROS) and cytoskeleton formation. The growth factor and cytokine -generated ROS are mediated by Rac, and the activation of Rac in the vascular wall is associated with atherosclerosis, myocardial hypertrophy, and endothelial dysfunction (169).

By inhibiting these (Rho and Rac) pathways, statins create non-cholesterol -dependent, pleoitropic effects that seem to be an important factor in cardiovascular protection. The positive effects have also been exhibited by inhibiting the GTPases individually, thus promoting cardioprotective effects such as endothelial function improvement or inhibition of atherosclerosis (276;332).

Statins affect eNOS expression and NO production in many ways. They affect eNOS expression by stabilizing its mRNA and reduce the binding of eNOS to caveolin-1, which as a complex reduces NO formation and activates the PI3K/Akt pathway, where Akt induces eNOS activation and NO production. Statins also restore eNOS activity in hypoxia and hypercholesterolemia (244;332).

Retrospective analysis of large statin trials, such as 4S, suggests that statins also reduce the incidence of heart failure (229). Recently two small prospective studies showed beneficial effects of statins in human heart failure. These patients showed improved cardiac function and reduced symptoms (248;331).

On the other hand, in the latest CORONA study, rosuvastatin failed to decrease mortality in elderly heart failure patients (228). The GISSI-HF study unfortunately displayed similar results, in patients treated with rosuvastatin (439).

Statins exert many effects beyond cholesterol lowering, which include diminished endothelial dysfunction, vascular inflammation, and inhibition of smooth muscle cell proliferation. These same molecular mechanisms can also be connected to kinins and their receptors, but there is no information concerning BK receptors and statins.

7.2 ACE and AT-Receptor Inhibitors

Angiotensin-converting enzyme inhibitors (ACEI) are effective anti-hypertensive agents, but also very potent in treating and preventing heart

failure. They are also powerful agents in treating and preventing MI, stroke, diabetes, and renal failure.

The protective effects of ACE inhibitors are due to their blocking the formation of Ang II and inhibiting the degradation of BK. Increased BK levels enhance NO synthesis and prostaglandin release through activation of BK-2Rs (232). ACEIs potentiate the effects of agonists on BK-2 receptors by inducing protein-protein cross talk between ACE and BK-2 receptors (283). They can also directly activate BK-1Rs (202). The antifibrotic effect of ACE inhibitors in heart failure are partly mediated by BK-2Rs (156). Many of the cardiovascular effects of ACEIs can be blocked by BK-2R antagonists or mimicked by exogenous BK, suggesting that the effects are mediated by the potentiation of bradykinin and/or BK-2Rs, rather than inhibition of Ang II conversion (232)

The effects of kinins with AT-1R inhibitors (ARBs) are thought to be mediated via activation of AT-2Rs. This activation seems to antagonize several effects induced by stimulation of AT-1Rs such as vasoconstriction, cellular growth, and hypertrophy (404). With AT-1R inhibitor treatment the increased concentrations of angiotensin II and its metabolites act on AT-2Rs and activate local kinin formation. They may also act on the kinin degrading enzymes to increase the tissue BK level (477).

Experimental studies show that kinins are also involved in the effects of AT-1R inhibitors. In an animal study where MI was induced to rats, losartan prevented LVH and HF and the effects were partly attenuated by concomitant inhibition of BK-2Rs (266). During the development of HF following myocardial infarction, BK-2R knockout-mice showed diminished improvement in cardiac function in response to AT-1R inhibitors than control mice (500). In mice that over-express AT-2Rs, infusion of Ang II induces hypotension, which can be abolished by icatibant (458).

The coupling between AT-2R and kallikrein during AT-1R inhibitor treatment and the increased plasma Ang (1-7) levels, which are known to potentiate the effects of bradykinin, may also contribute to the mechanisms related to AT-1R inhibitor-kinin cardioprotection (5;304).

Mounting evidence indicates that kinins are involved in the cardioprotective actions of ACE- and AT -receptor inhibitors the effects are mainly transmitted by BK-2Rs.

7.3. Anti-inflammatory Agents

7.3.1 COX -Inhibitors

Non-steroidal anti-inflammatory drugs (NSAIDs) block the activity of both COX isozymes, COX-1 and COX-2, which mediate enzymatic conversion of

membrane-derived arachidonic acid (AA) to prostaglandin H2 (PGH_2) and other prostaglandin (PG) metabolites: PGE_2, PGI_2, PGF_2, PGD_2, or tromboxane A_2 (TXA_2). These prostanoids have cardiovascular effects – they can act as either vasodilators or vasoconstrictors, they influence thrombosis formation via platelets, and they alter renal functions**.** The inducible isoform- COX-2 - is expressed at sites of inflammation such as atherosclerosis.

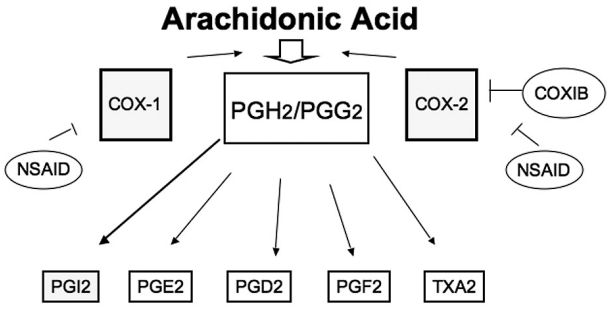

Fig. 10 The prostaglandin synthesis.

Prostaglandins are widely recognized as inflammatory mediators, but they can also regulate vascular contractility**.** COX-inhibitors are used as anti-inflammatory and pain relieving mediators. Cyclooxygenase-2 selective inhibitors (COXIBs) were developed with the prime objective of minimizing gastrointestinal adverse effects, which are seen with the use of traditional non-steroidal anti-inflammatory drugs (NSAIDs). During recent years several trials have connected COXIBs to unfavorable effects on cardiovascular outcomes and to increased risk of thromboembolic complications (334;421). At the same time there are controversial results suggesting that inhibition of COX-2 might be cardioprotective. To the latter belongs the fact that COX-2 can be detected from atherosclerotic lesions but not from normal coronary arteries (26). COX-2 knockout-mice develop myocardial fibrosis (111) and the expression of COX-2 seems to be up-regulated by atherosclerosis-inducing stimuli, including free radicals and increased arterial wall shear stress (3;492). And finally, because inflammation of the endothelium is known to diminish its NO -producing capacity, COX-2-inhibition was shown to improve NO production in CHD and hypertensive patients (83;482).

Despite the numerous studies, any conclusion about their cardiovascular safety is complicated due to the conflicting results.

Atherosclerosis is a process with inflammatory features, and selective cyclo-oxygenase-2 (COX-2) inhibitors may potentially have anti-atherogenic effects

by virtue of inhibiting inflammation. However, by decreasing vasodilatory and anti-aggregatory prostacyclin production, COX-2 antagonists may lead to increased pro-thrombotic activity (167)

The connection with the kinin system has been suggested earlier. BK is know to induce COX-2 expression in different cell types, such as endothelial and smooth muscle cells (54;55). Indomethacin, an unselective COX inhibitor, was able to cause potentiation of BK-induced contraction of both isolated rat uterus and guinea pig tracheal SMC preparations (80;398).

Down-regulation of BK-2Rs has been detected in rabbit hearts treated with a non-specific COX inhibitor, suggesting kinin-prostanoid-induced regulation of BK-2Rs (281).

It was recently also shown that BK induced COX-2 expression in aortic vascular smooth muscle cells, and the effect was mediated by BK-2Rs and involved MAPK p42/p44, PKC, and eNOS activation (379).

Clearly, there are interactions between kinin and prostanoid systems, and the involved kinin receptor seems to be BK-2R, but again, the specific mechanisms and clinical relevance need further studies.

8. BK- Receptors in Heart Failure

Considerable progress has been made in understanding the biology and function of bradykinin receptors. A large body of animal studies implicates a role for BK receptors in vascular pathophysiology. Little is directly known about the clinical relevancy of kinin receptor activation in humans, though an increasing amount of data indicates a cardioprotective role for kinin receptors in humans, also. Direct evidence of BK-receptor involvement in cardiovascular diseases is difficult to acquire, as the vascular segments needed for direct receptor observation are fairly difficult to obtain. Thus, the involvement of kinin receptors is mostly suggestive and based on receptor agonist/antagonist studies or detection of secondary pathway induction. Knockout-models have also provided valuable information about the regulation and function of BK receptors.

8.1 Lessons from Human Studies

8.1.1 BK-2 Receptors

In a prospective study with healthy army recruits, a ten-week physical training program, associated with LV growth response and BK-2R +9/-9 polymorphism and increased LV mass related to the +9/+9 genotype (60). The absence (-) of

a 9 bp repeat in exon 1 of the gene encoding the bradykinin 2 receptor is associated with higher receptor mRNA expression, higher gene transcriptional activity and lower cardiac trophic response to exercise training (56;60). Similar results were obtained from patients suffering from hypertension and LV hypertrophy. The +9/+9 genotypes responded worse than other genotypes to LV mass regression, independent of blood pressure reduction or treatment (174). There was also a strong link to ACE I/D polymorphism, suggesting a synergistic effect between kinin receptors and ACE. The results support the role for bradykinin in the regulation of left-ventricular growth (60).

Several studies associate BK-2R promoter region polymorphism -58 T/C and hypertension, where the C-allele is associated with LVH and/or hypertension at least in African-Americans, Japanese and Chinese subjects (141;155). African-Americans have a significantly increased prevalence, earlier onset, increased mortality in HF, and worse endothelial dysfunction, which could at least partly be explained by the 58 T/C polymorphism and the decreased activity of cardioprotective BK-2Rs. Also, a significantly higher incidence of the C allele of BK-2R was seen in hypertensive patients suffering from myocardial infarction in a Japanese study (18). There is no significant difference in the 58 T/C polymorphism, in the European population; the frequency of the C allele is 0.595 and for the T allele is 0.405, which are in Hardy-Weinberg equilibrium (56;174).

So far, the only human study on heart failure patients and BK-2 receptors was done a few years ago. In human end-stage heart failure patients, a decrease in BK-2Rs in both protein and mRNA levels was observed in the left ventricles of end-stage heart failure patients. There seemed to be no differences in cell-specific expression of BK-2Rs between normal and diseased hearts. The subsequent down-regulation of eNOS suggests a maladaptive connection between these two proteins (239).

The role of kinins in heart diseases has not received much attention, despite the fact that in the 1960s it was shown that both local and systemic administration of BK increased coronary blood flow and improved myocardial metabolism. It took almost 30 years to observe the same in humans. Infused BK doubled blood flow in the human forearm; the effect was abrogated by BK-2R antagonist icatibant (87).

The TREND (trial on reversing endothelial dysfunction) trail showed that ACE inhibitor treatment improved NO-mediated endothelial function in CHD patients. The results were confirmed in healthy and HF patients, where improved endothelial function was abolished by icatibant, indicating the significant role of kinins and BK-2Rs in the endothelium and cardiovascular diseases (192;193;278).

In atherosclerotic patients BK-induced vasodilation is impaired in the plaque area but preserved in spastic areas, suggesting that the EDHF might

have a role in vasodilation in CHD patients. These results were previously suggested in animal models (238;313;441).

A clear correlation between brachial dilation and epicardial dilation was established when bradykinin was infused into subjects with no signs of CHD, who were undergoing coronary angiography (290). Flow-mediated impaired dilation of the brachial artery is considered a non-invasive assessment of coronary endothelial dysfunction. Also in patients undergoing coronary angioplasty (PTCA), infusion of BK mimicked the effects of an ischemic precondition rendering the myocardium relatively resistant to subsequent ischemia (252).

Several studies have demonstrated that the serum of patients with heart failure can alter the function of endothelial cells by down-regulating the expression of endothelial nitric oxide synthase (7). Unfortunately, the role of BK-2Rs was not reported in these studies. ACE inhibitors improve endothelial vasomotor dysfunction in patients with coronary disease, and the effect is mediated by BK-2Rs (360).

Icatibant reduced the hypertensive effect of captopril (ACEI) in patients with hypertension by 53 percent (159).

8.1.2 BK-1 Receptors

Knowledge about BK-1Rs in human heart failure is scarce. Infusion of a promiscuous B1-B2-R antagonist (B9340) decreased forearm blood flow in HF patients taking ACE inhibitors. The effect was absent in patients with no ACE inhibitors as well as in a control group. Icatibant failed to dilate forearm blood flow, thus suggesting that BK-1Rs were mediating dilation in congestive heart failure concomitant with ACE inhibition (487).

In 1997 Raidoo *et al*. discovered that atheromatous arteries exhibit more intense BK-1R labeling in the endothelial cells, foamy macrophages, inflammatory cells, and fibroblasts within plaque and in the intima (364). This suggests that BK-1Rs are relevant in CHD. No other reports on this matter have been published since (364).

Two small studies recently showed that BK-1Rs did not have a major role in peripheral circulation in HF patients, nor could they show any cross-talk between BK-1 and BK-2 receptors (97;245).

The role of BK receptors in human heart failure is not convincing. Although there are numerous studies concerning kinins, knowledge about kinin receptors is weak. To complicate the issue, results from different animal studies have been somewhat controversial, which may at least partly be explained by the different species involved and the different expressions of receptors.

8.2 Lessons from Animal Studies

8.2.1 BK-2 Receptors

The number of BK-2Rs was dramatically decreased during the progression of heart failure in a pressure overload model in spontaneously hypertensive rats (SHR). The number of receptors first increased, correlating to the progression of LVH without fibrosis or ventricular dysfunction. Marked fibrosis and LV dysfunction were observed in the failing hearts, with a loss of BK-2Rs mainly located in the vascular endothelial cells, suggesting a role of the BK-2Rs in end-stage HF. Similar results were obtained from experiments in which acute pressure overload (aortic banding/Ang II infusion) induced the heart failure (240).

ACE inhibitors are the basis of medication in heart failure. Treatment with ACE inhibitors affects BK-2R regulation and potentiates its responses in many ways, not only by inhibiting kinin degradation (284;309). Numerous studies show that the positive cardiovascular effects of ACEIs are partly transmitted via BK-2Rs.

To elucidate the role of BK-2Rs, an orally active BK-2R antagonist (FR173657) was administered to dogs with HF under long-term concomitant treatment with ACEI.

Inhibition of BK-2Rs did not alter systemic blood pressure or LV chamber dimension. Increased LV filling pressure and suppressed expression of eNOS and sarcoplasmic reticulum Ca^{2+}-ATPase mRNA were detected as well as up-regulation of collagen I and III expression. Collagen deposits were increased in the myocardium of the failing hearts in the BK-2R antagonized group, indicating a detrimental role for BK-2Rs in the progression of HF. The treatment did not alter systemic blood pressure or vascular resistance in HF dogs, suggesting also that endogenous BK does not participate in blood pressure regulation via BK-2Rs (156).

BK inhibited the progression of cardiac hypertrophy and myocardial fibrosis in hypertensive rats due to activation of BK-2Rs (437).

Chronic infusion of bradykinin was also able to preserve ventricular and endothelial function in conscious dogs, while delaying the progression of heart failure (447).

The role of BK-2Rs in ischemic preconditioning has been observed in several studies.

The positive effects are reduced infarct size and elimination of post-ischemic arrhythmias by icatibant in preconditioned rats, rabbits, and mice. The positive preconditioning effects are not evident in BK-2R knockout mice nor in kininogen -deficient Brown Norway rats, indicating the significance of the kinin system to cardioprotection in I/R injury (468;501).

BK seems to have a preventive role in the progression of HF, and the effects seem to relate to activation of BK-2Rs and involve NO and also the EDHF, both induced by BK-2Rs.

8.2.2 BK-1 Receptors

BK can exert cardioprotective effects via BK-1Rs, which are known to be involved in the BK-induced reduction of catecholamine release in ischemic rat hearts and in the maintenance of endothelial function in preconditioned hearts (53;77).

There are controversial results concerning the regulation and effects of BK-1Rs in myocardial ischemia. Contradictory results concerning the cardioprotective role of BK-1Rs in myocardial ischemia have been obtained from BK-1R KO-models (242;249;493).

Myocardial sympathetic fibers express BK-1Rs and release noradrenalin. BK inhibits noradrenalin release in a BK-2R dependent way in ischemic rat hearts.

This inhibition was only observed in the presence of BK-1R antagonists, suggesting that BK-1R activation, induced by hypoxia, is involved in myocardial NA release (77;152).

9. Aims of the Study

The general hypothesis of the study was that the kinin receptor system, notably BK-2Rs and BK-1Rs, plays an important role in the pathophysiology of left ventricular hypertrophy and participate in the pathogenesis and progression of heart failure.

The aims of the study were to investigate the regulation of kinin receptors in normal and failing heart tissues, and in particular, in coronary artery endothelial cells. More specifically the aims were:

1. To clarify the expression of BK receptors in normal and failing human heart tissue.
2. To compare the accumulation of two known BK-2R gene polymorphisms affecting gene expression in normal and failing human hearts.
3. To investigate the regulation of BK-receptors in human coronary endothelial cells by both physiological and pharmacological factors, including hypoxia and lovastatin.

10. Materials and Methods

The biochemical methods central to this thesis and their use in the original publications are summarized in table 2.

The methods are only briefly described here, and detailed descriptions with references are provided in the Methods sections of the original publications.

Table 2. Methods used in the original publications

Method	Publication
Preparation of human heart samples	I, IV
RNA isolation	I, II, III, IV
Competitive RT-PCR	I, III
Real-time RT-PCR	II, III
Protein extraction	I, II, III
SDS-PAGE gel electrophoresis	I, II, III
Culture of human endothelial cells	I, II, III
Isolation of rat endothelial cells	III
Culture of rat micro vascular endothelial cells	III
Histological staining	I, III
Immunocytochemistry	II, III
cGMP immunoassay	II
NO measurement	II, III
"Wound healing" assay	III

Human Heart Samples (I, IV)

Normal heart samples (n=6) were obtained from the left ventricles of organ donors who had no history of cardiac disease and had been excluded from organ donation because of age, body size, or tissue-type mismatch. The cause of death in these subjects was subarachnoid hemorrhage. The failing left ventricles were harvested at the time of cardiac transplantation from 13 patients with end-stage heart failure (New York Heart Association functional class IV) due to either idiopathic dilated cardiomyopathy (IDC; n = 7) or coronary heart disease (CHD; n= 6) at the University Central Hospital, Helsinki, Finland. All the patients had been treated with a combination of drugs, including beta-blockers, ACE inhibitors, loop-diuretics, digoxin, and spironolactone. None of the patients had received angiotensin II type I receptor blocker treatment or statin treatment. After excision, the heart tissues were immediately frozen in liquid nitrogen and stored at –70 °C. The left ventricle myocardium devoid of visible scar tissue was used in the experiments. An

institutional review board approved the use of normal and failing human heart samples, and the investigation conforms to the principles outlined in the Declaration of Helsinki.

RNA isolation and PCR (I-IV)

Total RNA was isolated from both hearts and cell cultures using an ultrapure TRIzol reagent (GIBCO-BRL) and a RNeasy Mini Kit (QIAGEN). Purified total RNA (0.25 μg for RT-PCR and 1 μg for real-time RT-PCR) was transcribed into cDNA using a Superscript preamplification system (GIBCO-BRL).

Competitive RT-PCR was performed in 25 μl of a standard PCR buffer containing 1 μl of the RT reaction mixture, 25 pmol of sense and antisense primers, 100 μM of each deoxynucleotide, 0.5 units of *Taq* DNA polymerase (Roche), and competitor DNA. The primers used were BK-1R, BK-2R, TNF-α, and GAPDH as the housekeeping gene. The competitor DNAs for BK-1R, BK-2R, and TNF- α were obtained by subcloning the obtained PCR fragments and inserting a 129-bp external DNA fragment into the *Sma*I, *Sac*I, and AvaI sites of the respective PCR fragment. The PCR fragments were verified to represent the corresponding targets either by specific restriction enzyme treatment or by DNA sequencing. The PCR products were quantitated with a gel documentation system (Gel Doc 2000; Bio-Rad, Hercules, CA), and when a competitive method was used, the logarithm of target-to-competitor ratio was plotted against the logarithm of the competitor DNA molecules.

For real-time RT-PCR the DNA and mRNA sequences of the target genes were retrieved from the GenBank® database (National Institutes of Health, Bethesda, MD) and gene-specific primers and fluorogenic probes were identified with the Primer Express® software package. The primers used were BK-1R, BK-2R, and GAPDH as the endogenous control. The analyses were performed in 96-well plates (Applied Biosystems) using a TaqMan© Universal Master Mix with uracyl-N-glycosylase treatment in 25 μl reaction volumes. They were analyzed with an ABI Prism 7500 Sequence Detection System using 45 cycles of a two-step program consisting of 15 s at 95 °C and 1 minute at 60 °C. The threshold was set to the geometric phase of the amplification curve, and the amount of target was calculated using the $2^{-\Delta\Delta C}$T formula.

Protein Extraction and Western Blotting (I-IV)

Triton X-100 extracts were prepared from heart tissue and cell cultures, subjected to SDS-PAGE, and electro blotted onto nitrocellulose filters (261).

The proteins were detected by immunoblotting using antibodies listed in Table 3. The primary antibodies were further detected with horseradish peroxidase-labelled secondary antibodies and enhanced chemiluminescence

(ECL) as described by the manufacturer (ECL, Amersham Pharmacia Biotech). The translation products were quantitated with a gel documentation system (Gel Doc 2000, Bio-Rad).

Table 3. List of antibodies used

Primary Antibody	Host	Isotype	Source
BK-1R	rabbit	Polyclonal	Santa Cruz
BK-1R	rabbit	Polyclonal	Own product ref.(364)
BK-2R	mouse	Monoclonal	Transduction Lab
TNF-α	mouse	Monoclonal	Santa Cruz
TNF-R1	mouse	Monoclonal	Santa Cruz
β-actin	mouse	Monoclonal	Abcam
RhoA	mouse	Monoclonal	Santa Cruz
Control	mouse	Monoclonal	Serotec

Histological and Immunocytochemical Staining

The frozen human and rat hearts were dissected for immunohistochemical stainings using a standard immunoperoxidase method. Briefly, the slides were fixed with ice-cold methanol. Endogenous peroxidise activity was blocked by means of incubation in 2% H_2O_2 in PBS for 10 minutes. After washing the slides carefully, they were blocked for 60 minutes with 3% normal serum in order to avoid unspecific binding of antibodies. The slides were subsequently incubated with primary antibodies and diluted in corresponding blocking serum overnight at +4 °C. The slides were then washed and a secondary antibody was applied. The nuclei were stained with 4´, 6-diamino-2-phenylindole dihydrochloride (DAPI) or with hematoxylin and after washing the mounting media- fluorescent or not- added and examined under a normal or fluorescent microscope, depending on the antibodies used.

The samples subjected to immunohistochemical staining, were stained using primary antibodies listed in Table 2. The cultured endothelial cells were cultured on coverslips until subconfluent. The cells were incubated in the absence or presence of different substances (BK 10-100nM, Icatibant 1μM, lovastatin 1nM-100μ, mevanololactone 100μM, Y27632 10μM) or in hypoxic conditions. After this the cells were fixed with 4% paraformaldehyde and kept at +4 °C until staining. The primary antibodies were incubated overnight, carefully washed, and stained with secondary antibodies. The immunostained cells were analyzed using fluorescent microscopy (Nikon Eclipse E6000 microscope).

Culture of Human Coronary Arterial Endothelial Cells

Human coronary artery endothelial cells (HCAEC) were purchased from Promo Cell GmbH and used for experiments during passages 4–6. The cells were grown in culture media supplemented with growth factors as recommended by the manufacture.

The cells were incubated in a humidified incubator at 37 °C with a 5% CO_2 atmosphere. The medium was changed every second day and experiments were performed with subconfluent cultures. For the hypoxia experiments, the cells were changed into a low-oxygen containing incubator (1% or 5% O_2) and grown there for the needed time.

Isolation and Culture of Rat Coronary Artery Microvascular Endothelial Cells

Male Wistar rats (300–500 g) were obtained from the Laboratory Animal Center of the University of Helsinki. The rats were treated in accordance with institutional guidelines, which had been approved by the institutional Ethics Committee. The rat myocardium was dissected for perfusion with an enzyme solution for 20 minutes and supplemented with BSA for an additional 10 minutes to facilitate the recovery of separated cells. The perfused tissue was cut into small pieces, incubated with the enzyme solution, and then filtered. The myocardial perfusate and suspension were combined and treated with trypsin, $CaCl_2$, and BSA in the perfusion buffer for 30 minutes. The cells were collected by centrifugation and resuspended in a culture medium supplemented with FBS and appropriate growth factors and antibiotics. The cells were identified as RCMCs and cultured in M199 growth media at 37 °C under 5% CO_2. The experiments were performed with ECs of 2^{nd}-6^{th} passages in serum-free conditions.

Measurement of NO in Human Coronary Arterial Endothelial Cells

NO production in cultured HCAECs was studied by using 4,5-diamino-fluorescein diacetate (DAF-2-DA) as a fluorescent indicator of intracellular NO, as previously described (462). Briefly, the HCAECs were grown on glass coverslips and treated in the presence or absence of lovastatin or in hypoxic conditions. The HCAECs were pre-incubated for 30 min with L-arginine, pulsed for 10 minutes with DAF-2DA, and washed with PBS. The cells were resuspended in PBS containing L-arginine and activated for 10 minutes with BK. Intracellular fluorescence was visualized with a Nikon Eclipse E600 fluorescence microscope using excitation at 495 nm and emission at 515 nm.

cGMP Immunoassay

cGMP was analyzed with the direct cGMP Enzyme Immunoassay Kit (Assay Designs, Ann Arbor, MI) according to the protocol described by the manufacturer. Briefly, lovastatin was added to the subconfluent plates and incubated with the cells to obtain a high level of functional BK-2Rs in the HCAECs. The lovastatin-treated HCAECs were activated with BK for 30 or 60 minutes after which the cells were washed and endogenous phosphodiesterase activity was inhibited. The cells were lysed with 0.1 M hydrochloric acid and the cell lysate was subjected to overnight acetylation and then used in the competitive cGMP EIA kit as recommended by the manufacturer. The results were analyzed with a microplate reader (Victor^{3_3}, Perkin Elmer) at 405 nm.

Wound-healing Assay

An *in vitro* wound-healing assay was performed as previously described by Buzza *et al*. (64). The isolated rat coronary microvascular endothelial cells (RCMEC) were cultured on top of thermanox slips in a culture media at +37 °C in a humidified incubator with 5% CO_2. After 2 hours of incubation, non-attached cells were removed by washing. The cells were cultured until subconfluent; an endothelial wound was applied to the slip with an appropriate sized scrape. The treated slips were then carefully washed to remove detached RCMECs and fresh culture media was added in the absence or presence of bradykinin and/or icatibant. The thermanox slips were then incubated under mild hypoxic culture conditions (5% O_2) for 24 or 48 hours to induce BK-2R expression, and fresh BK and/or icatibant was added at the begining and after 24 h of incubation. The experiment was terminated by washing the slips 3 times with ice-cold PBS (including Ca^{2+}/Mg^{2+}) and fixed with ice-cold 4% paraformaldehyde (PFA). The fixed RCMECs were washed and the cells were stained with hematoxylin and photographed with a digital camera attached to a Nikon Eclipse E600 microscope.

- / + 9 Polymorphism in Exon 1 of the BK-2 Receptor Gene

Genomic DNA was isolated from human heart tissue using an RNA/DNA Mini Kit (Qiagen) and stored in TE buffer (pH 8.5) in aliquots at –20 ℃. Isolated genomic DNA was subjected to PCR using primers spanning the insertion/deletion (-/+ 9 bp) site in exon 1 of the BK-2R gene. The PCR was run at T_m = 66 ℃ for 40 + 1 cycles, and the obtained PCR products were separated on a 3% MetaPhor agarose gel (BMA).

-58 T/C BK-2 Receptor Promoter Polymorphism by PCR and SSCP

The genomic DNA was subjected to RT-PCR. The obtained PCR products were denatured by heating at 94 ℃ for 3 minutes in 95% formamide, 10 mM NaOH, 0.25% bromphenol blue, and 0.25% xylene cyanol, and then rapidly cooled on ice. The denatured PCR samples were mixed with 9 parts of SSCP Sucrose Dye and run on a 14% acrylamide/bis (29:1) gel (Bio-Rad). The gel was stained with SYBR Gold nucleic acid gel stain (Molecular Probes) for 30-40 minutes and analyzed with a Gel Doc 2000 gel documentation system (Bio-Rad).

11. Statistical analysis

Data are expressed as means +/-SEM. The patient groups showed normal distribution when analyzed with the Shapiro-Wilks W-test. Correlation studies were performed by using Pearson's correlation and/or ANOVA statistics. Comparisons between the groups were made by using Student's *t-test*. Statistical significance was accepted as $P < 0.05$.

12. Results

BK-1 Receptors in Human Heart Failure

A series of end-stage HF human hearts were analyzed in regard with BK-1Rs and compared with control hearts.

A significant increase in BK-1Rs in both mRNA and protein levels in the diseased hearts was discovered. The BK-1R mRNA expression was increased 2.1 -fold in CHD patients and 2.8 -fold in IDC patients compared with control hearts.

A low transcript level of BK-1R mRNA was also detected in normal myocardias. This was further confirmed by immunohistochemical staining, where a weak staining was detected in intramyocardial vessels also in normal hearts.

The protein quantities of BK-1Rs were also more abundant in HF hearts compared with control ones. The protein levels were somewhat higher in CHD hearts than in IDC hearts, indicating that BK-1Rs are regulated at both transcriptional and translational levels, which has previously been suspected in HF with other genes (240;428). Moreover, the BK-1R/BK-2R mRNA ratio was calculated and there seems to be clear differences between the hearts, thus in the normal myocardias 79.000 copies, 32.000 copies in CHD and 25.000 copies/ 1μg RNA in IDC heart samples were detected.

Intense staining of TNF-α was detected in the diseased hearts and the

expression of TNF- α correlated with BK-1R expression in Pearson's correlation test. The immunoreactivity of TNF-α was strongly labelled in the intra-myocardial vessels and specifically in the endothelial cells, as were also the BK-1Rs. Some staining was also detected in the interstitium and in the myocardial cells. Furthermore, incubation of human coronary endothelial cells with and without recombinant TNF-α, and showed, that BK-1R expression was accelerated with the introduction of TNF-α at both the mRNA and protein levels.

TNFR-1 staining was intense in the intra-myocardial vessels of the diseased hearts and the receptors co-localized with the BK-1Rs in the vessel endothelial cells.

BK-2 Receptor Correlation with Age and Polymorphism

The BK-2R mRNA expression was analyzed and a linear regression analysis was done, comparing age and receptor amounts in the control hearts. A clear positive correlation was found, between age and BK-2R mRNA expression, suggesting that transcription of BK-2Rs increases with age in healthy hearts. A positive correlation between age and BK-2R mRNA was also found in the IDC hearts, but a slightly negative correlation in CHD hearts.

Previously it was suggested that BK-2Rs were regulated on both the transcriptional and translational levels in human heart failure (239). By plotting the BK-2R levels against the protein levels from both normal and failing hearts, a positive, linear correlation was found between the mRNA and protein levels, suggesting that BK-2Rs are regulated on the transcriptional level and not on the translational level, as previously suggested.

In the promoter region polymorphism -58 T/C, a marked genotypical frequency of CC allele was found in the IDC hearts. In IDC hearts 5/7 possessed genotype CC (71%) compared with 2/6 of this allele (33%) in normal hearts. Equal quantities of the TT allele were found in IDC and normal hearts, 1/7 and 1/6 (14% and 17%). The dominating genotype in normal hearts was TC, seen in 3/6 (50%). The genotypic frequencies in the CHD hearts were similar to those in normal hearts. furthermore, the allelic frequencies for -58T/C polymorphism were 0.58 for the C-allele and 0.42 for the T-allele in normal and CHD hearts and 0.79 and 0.21, respectively, in the IDC hearts.

For the -9 bp deletion/insertion SNP in exon 1, all the CHD hearts (100%) were homozygous for this deletion (-9/-9). In normal hearts only 1/6 (17%) and 2/7 in IDC hearts (29%) were homozygous for this deletion. Seventy-one percent of the IDC hearts (5/7) were heterozygous (-9/+9) for the deletion and of the normal hearts, 3/6 (50%) were homozygous for the insertion in the allele (+9/+9). See Table 4.

Table 4. Characteristics of human hearts

Subject No.	Etiology	Sex	Age	Polymorphism	
				-58 T/C	+9/-9
1	Normal	M	19	T/C	+9/-9
2		M	43	T/C	+9/+9
3		M	49	C/C	+9/-9
4		M	31	T/T	-9/-9
5		M	40	T/C	+9/+9
6		F	50	C/C	+9/-9
7	CHD	M	61	T/C	-9/-9
8		M	60	T/T	-9/-9
9		M	55	C/C	-9/-9
10		M	52	T/C	-9/-9
11		M	60	C/C	-9/-9
12		M	54	T/C	-9/-9
13	IDC	M	29	C/C	-9/-9
14		M	60	C/C	-9/-9
15		M	46	C/C	+9/-9
16		M	56	C/C	+9/-9
17		M	55	T/T	+9/-9
18		M	56	T/C	+9/-9
19		M	63	C/C	+9/-9

Induction of BK -Receptors by Lovastatin in Cultured Endothelial Cells

Incubation with lovastatin up-regulated BK-2R mRNA expression in cultured human endothelial cells (hCAEC). A dual curve was presented regarding the induction of BK-2R expression. At first a rapid and significant (> 11x) augmentation of the receptor mRNA was detected after 12-hour incubation with lovastatin. The BK-2R receptor expression mRNA declined back to the baseline after 24-hour incubation, where after incubating the ECs with a concentration of 100nM lovastatin, increasing levels of BK-2R expression was presented in the cultured cells during 72 hours of incubation. The level of BK-2R mRNA was approximately five-fold greater than in the untreated control cells after 72-hour incubation.

Incubating the cells with lovastatin also induced the expression BK-1R mRNA. Already, after 6-hour lovastatin incubation the BK-1R expression was increased over 23-fold. Also the level of BK-1R expression was declined back to control levels during 24-hour incubation. The expression level of BK-1Rs also showed a tendency of induction, during the prolonged (72 h) incubation of cells with lovastatin, reaching a seven-fold expression level compared with the control cells, not incubated with lovastatin.

The increased BK-2 receptor mRNA transcripts were translated into protein, shown by Western-Blotting analysis, as the protein levels were also up-regulated. The staining of BK-2 receptors was also clearly more intense in endothelial cells treated with lovastatin compared to control EC cultures.

The receptor induction was concentration-dependent and the greatest up-regulation was obtained with a fairly low lovastatin concentration (100 nM). The receptor inducing effect was abolished with higher concentrations (> 1 μM) of lovastatin, and the highest concentrations of lovastatin were actually toxic to the endothelial cells.

To establish the functionality of the inducted receptors, two indirect methods were used to show production of NO in lovastatin treated cultured hCAECs.

First, in cultured endothelial cells, incubated with lovastatin and at the presence of BK, the production of intra cellular cGMP was significantly increased. The induction required the presence of BK and lovastatin, thus in cells not boosted with BK, or incubated with lovastatin, only a slight elevation of cGMP production was detected.

Secondly, the increased production of NO was verified by a fluorescent DAF-2DA stain. Again, incubating the cells with lovastatin and exogenous BK, the measured fluorescence increased in a BK concentration-dependent way. The increased production of NO was estimated as three times greater than in cells not pulsed by BK.

The fluorescence also markedly intensified with increasing the concentration of lovastatin.

Mevalonate completely abolished the BK-2R induction in lovastatin treated cells, indicating that downstream mediators of the cholesterol pathway were involved in the induction of BK-2Rs.

Lovastatin induced the amount of RhoA protein in cultured endothelial cells but prevented the activation of RhoA, which was shown by immunocytochemical stainings. This was confirmed by incubating the cells with a specific inhibitor (Y-27632), of Rho-associated proteins (ROCK). By inhibiting ROCKs, a time-dependent increase in the expression of BK-2R mRNA was detected, which was similar to the results presented with lovastatin treated cells.

Incubating the ECs together with lovastatin and a specific COX-2 inhibitor (NS398), the BK-2R induction was abrogated. The incubation of mere COX-2 inhibitor had no effect on the BK-2R expression.

Induction of BK-2 Receptors in Hypoxia

In mild hypoxic conditions (5% O_2) the level of both BK-2R mRNA and protein expression was significantly induced compared with cells grown in normoxic conditions. The amount of BK-2R mRNA was time-dependently induced in mild hypoxic conditions. In contrast, severe (1% O_2) hypoxia clearly reduced the BK-2R expression.

The hypoxic induction was abolished by incubating the cells with icatibant, a specific BK-2R antagonist. The protein expression was also induced in mild

hypoxic conditions and where icatibant again abrogated the BK-2R induction.

Functionality of the receptors was verified by DAF-2DA immunocytochemical florescent stainings. In hypoxic cells the increased NO production was shown to be approximately three times greater than in normoxic cells. The NO production was induced in hypoxic cells but a clear difference was also detected in cells incubated with or without additional BK in mildly hypoxic chamber, where additional BK increased the amount of NO detected.

These *in vitro* results were then compared with *in vivo* rat experiments. Rats subjected to myocardial infarction by LAD ligation, showed an increased number of BK-2Rs in the endothelium of the myocardial vessels at the infarct border zone, suggesting that the BK-2Rs were also induced *in vivo* by hypoxia. To address this hypothesis, a "wound healing" assay was conducted, previously described by Buzza *et al.* (64)**.** This experiment showed that hypoxic conditions induced endothelial cell migration, which was further augmented by additional BK. The migratory effects were almost abrogated by incubating the hypoxic cells with icatibant. Proliferation of the migrating cells was also detected, thus the cells expressed Ki-76, a commonly used proliferation marker (data not shown).

Hypoxia induced functionally active BK-2Rs in a BK-K-2R-NO -dependent way. The induction of migration and proliferation in hypoxia was increased by BK and abrogated by icatibant.

The main results

In human end-stage HF:

- The BK-1R expression is increased in the intra-myocardial vessels.
- The BK-1R induction is accompanied with TNF-α induction and TNFR-1 co-localized with BK-1Rs.
- The expression of BK-2Rs increases with age in healthy human myocardias.
- The CC -allele of -58 T/C BK-2R SNP is more abundant in IDC hearts, compared to normal or CHD hearts.

In cultured endothelial cells:

- Lovastatin treatment increases the expression of functional BK-2Rs in a time and concentration dependent way. The induction can be abrogated by mevanolate.
- The induction of BK-2Rs in statin treated cultures is also inhibited by a COX-2 inhibitor, NS398.
- Hypoxia induces BK-2R expression in cultured cells. The induction of receptors, NO production but also angiogenesis was clearly increased in hypoxic cultures pulsed with BK. Icatibant abrogated all these effects.

13. Discussion

In the present study, I have focused on understanding the function and regulation of BK receptors in the pathophysiology of human heart failure. HF is a clinical syndrome caused by different cardiovascular diseases and the kinin system seems to be implicated in its pathogenesis. Kinins are powerful bioactive autacoids that have been shown to reduce the myocardial preload and oxygen consumption, thus improve the cardiac metabolism and function. Kinins exert their biological functions by activating specific kinin receptors. In particular, BK activated BK-2Rs are widely accepted as cardioprotective molecules, as they mediate both vasodilatory, antifibrotic, and antiproliferative effects via an increased production of NO, EDHF and PGI_2. BK is also known to counteract endothelial dysfunction, a phenomenon involving a decreased expression of eNOS with subsequent reduction in NO bioavailability, present in the hypoxic myocardium during HF.

The role of BK-1Rs in heart diseases and especially in HF has remained vague. The differences in kinin signaling in normal and failing hearts may depend on either kinin concentrations, kinin degrading enzymes, or on the regulation of BK-receptors, that may account for kinin mediated effects in endothelial cells, myocytes, and fibroblasts.

BK-1Rs and Inflammation

Kinins are proinflammatory peptides acting as local hormones and kinins induce the release of endothelium derived relaxing factors. Many of these effects are mediated through activation of BK-2Rs but in pathological conditions, also BK-1Rs mediate the cardiovascular responses of kinins.

Various studies show an induction of BK-1Rs in pathological conditions, such as tissue trauma, inflammation, or anoxia. Indeed, it has been suggested that BK-1Rs partly account for the initiation of inflammation (132;242). Moreover, the level of inflammatory cytokines is increased in HF, which are known to induce the expression of BK-1Rs (256). The role of BK-1Rs in cardiovascular diseases derives mainly from animal studies and has remained controversial (53;242;292).

In the present study, we showed an increased expression of BK-1Rs in human end-stage failing hearts, implying that induction of BK-1Rs reflects the pathological progression of HF and ongoing inflammation. Previous studies in hypertensive rats show that up-regulation of BK-1Rs seems to be associated with the transition from compensated LV hypertrophy to heart failure, with the onset of myocardial fibrosis and with the development of diastolic dysfunction (240;452).

In previous study, Kuoppala *et al* showed low levels of BK-2Rs in the

LV of end-stage HF patients and the study detected a significant increase in fibrosis in IDC hearts compared with normal hearts (239). In the present study we showed increased levels of BK-1Rs, as well as an increased ratio of BK-1R/BK-2R in the myocardium of the HF hearts, suggesting that inflammation accompanies the increased level of fibrosis in these failing hearts. This has previously been addressed in human lung fibroblasts, where induction of BK-1Rs activated fibrogenesis. Moreover, in stenotic aortic valves, a significant induction of BK-1Rs was detected in the diseased valves compared to control valves. Myofibroblasts, cultured from the stenotic valves, were also more susceptible towards TNF-α induced BK-1R up-regulation (181;293).

Our study also showed a correlation of the increased TNF-α levels and TNFR-1 expression in the intra-myocardial coronary vessels with induced BK-1R levels as well as co-localization of BK-1 and TNFR-1s. The results imply that an inflammatory context in HF is a regulator element in BK-1Rs expression.

Whether the upregulation of BK-1Rs in the failing human hearts reflects increased production of cytokines or a compensation of the BK-2R down-regulation can presently not be answered. However, knockout studies have shown that BK-1Rs are up-regulated in the heart of BK-2R knockout-mice and they can takeover some of the hemodynamic properties of BK-2Rs, suggesting that the BK-1Rs can take a compensatory role at least in the absence of BK-2Rs, and thus be cardioprotective (124).

The highest level of BK-1Rs and the lowest of BK-2Rs were detected in IDC hearts, suggesting that the etiology of HF and BK-receptor regulation are interrelated. The observation that the level of fibrosis in the end-stage HF patients was higher in the IDC hearts compared to CHD hearts supports this notion (239). The differences in receptor levels between CHD and IDC may also be explained by differences in the genetics, a notion supported by previously observed differential expression of several genes in coronary arteries from CHD and IDC patients (388).

Deletion of BK-1Rs does not, under basal conditions, affect cardiovascular function or growth and BK-1R knockout-mice do not develop hypertension or myocardial hypertrophy nor HF (242;352), suggesting that BK-1Rs have a diminutive role on cardiovascular regulation under normal situations. On the other hand, animal models and human artery *in vitro* studies have shown that BK-1Rs can regulate vascular tone (230;286;487). Under healthy conditions, the level of BK-1Rs is undetectable or extremely low in human myocardias. Our perception of a low BK-1R level also in control hearts, suggests that BK-1Rs may exert some function in the normal cardiovascular regulation. It may also merely reflect a normal aging process, previously suggested in animal studies (225).

BK-2Rs and Aging

In our study, the level of BK-2Rs correlated positively with age in healthy hearts, suggesting that healthy human heart adapts to ageing or senescence by inducing BK-2R expression.

The association between BK-2Rs and senescence has been recently reported in bovine endothelial cells, where BK dose dependently protected the cells from ROS induced senescence through pathways involving BK-2R and NO (336). In addition, in a report from Kakoki *et al*, BK-2R knockout-mice displayed a slightly accelerated ageing phenotype. The ageing phenotype was strongly promoted when the BK-2R knockout was combined with diabetes (212). These results strongly suggest, that BK-2Rs are associated with senescence and protect against its damaging effects.

We also reported a positive correlation between BK-2R mRNA and age in IDC hearts, where as the correlation was negative in CHD patients. This can reflect the some what older patient material of the CHD group, but may again also imply disease-dependent regulation of BK-2Rs or a genetic variation.

Contradictory results were reported from animal studies, where unchanged levels of BK-2R mRNA were detected, from young and old rats. Whether this reflects normal ageing process in rats or differences in cellular composition in ageing hearts can not be answered. A discrepancy was detected in the study by Kintsurashvili *et al,* between the gene expression and protein translation, thus in old rats the BK-2R protein levels were two-fold lower as compared to young ones (225). The differences between gene expression and protein translation in the study suggests, that BK-2Rs are regulated post-transcriptionally, which was also our finding in the end-stage human hearts.

BK-2 Receptor Polymorphism

Racial differences in survival outcomes, point to genetics in the predisposition of HF and to date two BK-2R polymorphisms have been connected to hypertension and HF (119). In our studies the C-allele in -58 T/C SNP was significantly more abundant in IDC patients which could account for the development of HF and also the lower level of BK-2Rs detected in IDC patients. The age-related up-regulation of BK-2Rs was impaired in IDC hearts, suggesting that high frequency of the C-allele, detected in IDC hearts, might relate to the lower BK-2Rs levels detected.

As for the +9/-9 insertion/deletion SNP, all of our CHD hearts possessed the -9 deletion. None of the diseased hearts and 33% of the control hearts were homozygous for the +9 insertion, suggesting that the known genetics of +/-9 was not associated with down-regulation of BK-2Rs in our patients.

BK-2Rs and Statins

As the endothelium is the major contributor of kinin receptor function, we have also studied endothelium and factors modulating kinin receptors in endothelial cells. In many clinical studies, statins have reduced mortality in CHD patients. Subclass analyses have suggested that statins might also be beneficial in HF patients without CHD, though these results have not been confirmed.

Our novel results show that lovastatin is able to induce both BK-receptors types in cultured human endothelial cells. The induction of BK-2Rs is concentration-dependent and clinically-relevant doses (lovastatin 80mg) seem to correlate with the greatest BK-2R induction. The effects were abrogated by mevanolate, implying that the down-stream mediators of cholesterol biosynthesis pathways are involved. More specifically, it involved inhibition of RhoA-Rho kinase-mediated downstream signaling pathways, previously been shown to have an important role in cellular functions in the pathogenesis of cardiovascular diseases. Rho-kinase up-regulates several molecules that accelerate inflammation and fibrosis, and down-regulates eNOS (247;434).

The present study showed that exogenous BK accelerated the up-regulated BK-2R expression and increased NO production in lovastatin-treated human ECs. A direct regulatory link between BK-2R and eNOS has been shown, where the level of eNOS and also production of NO depend on the level of BK-2R. (23;509).

In a dog model BK induced NO production in renal and coronary arteries, which was attenuated with HF dogs and retrieved by simvastatin.(4;451). Moreover, the BK induced relaxation in rat mesentery arteries was enhanced by simvastatin, which resulted from inhibition of RhoA and attenuation of the vascular contractility through augmenting the endothelial NO production in mesenteric arteries (408). Unfortunately, no data concerning BK receptors was reported.

By reducing the prenylation of isoprenoids, statins inhibit Rho–kinases, accelerated by several inflammatory stimuli such as IL-1β and angiotensin II (186). In our studies, the statin effect on BK-2Rs was reversed by both mevanolate and icatibant and mimicked by Rho inhibitor, indicating the involvement of Rho in the signal transduction leading to BK-2R expression.

The results suggest that BK increases NO production via activating BK-2Rs and eNOS in statin-treated endothelial cells. This involves the RhoA-Rho-kinase pathway, thus implying that some of the pleoitropic effects of statins may be explained by induction on BK-2Rs, at least in endothelial cells.

The relevance of BK-1R induction by lovastatin remains unclear. Statin treatment attenuates inflammatory signaling pathways, implying another mechanism for the induction of BK-1Rs, in statin treated cells.

It has previously been shown in a murine model that ACE inhibitors can up-regulate functional BK-1Rs, suggesting that also BK-1Rs have beneficial

cardiovascular effects in the BK-BK-R signaling cascade, without inflammation (286). Moreover, deletion of BK-2R has been shown to induce the expression of BK-1Rs, suggesting a role for these receptors in the cardiovascular regulation (213).

Conflicting results were reported recently, where disruption of BK-2Rs resulted in early development of LVH and down-regulation of eNOS. The effects were abrogated by simvastatin and the authors concluded that simvastatin reversed the effects by activating the myocardial eNOS and MAP kinase (339). Moreover, no differences in BK-1R expression were detected between wild type or BK-2R knockout mice nor had simvastatin treatment any effect on the BK-1R expression. The discrepancies between these studies can relate to differences in species studied, to different knockout variants used or differences in experimental set-ups.

The exact mechanisms, by which statins induce BK-receptors, is presently not known, although a direct gene activating effect may be involved. In the light of former studies our *in vitro* results suggest that in heart failure, patients might benefit from statin treatment through induction of BK-2Rs resulting in increased NO production.

BK-2Rs and COX-2

Of the two known COX-isoforms, COX-1 is traditionally viewed as the constitutive enzyme, responsible for the production of the physiological amounts of prostaglandins needed. COX-2 is induced by various inflammatory stimuli and cytokines, and considered as a mediator of inflammation and pain.

COX-2 expression can be found both in the vascular ECs and SMCs and in the ECs, it mainly forms prostacyclin (PGI_2), which induces vasodilation. Prostacyclin is an important secondary mediator, often released by kinins from the endothelium, which stimulates cyclic AMP in vascular smooth muscle cells leading to vasorelaxation. Prostanoid formation occurs in many cell types, which possess kinin receptors.

Statin treatment has previously been shown to inhibit the generation of COX-2-derived prostanoid production and also down-regulate COX-2 expression in both endothelial and vascular smooth muscle cells (98). Recently several opposing reports have been published, where statins induce COX-2 expression and PGI_2 formation in human endothelial cells through inhibition of Rho (105;288).

In our study, the induction of statin treatment on BK-2R expression was reversed by COX-2 inhibitor (NS398). Moreover, no effect on BK-2Rs was detected when incubating the cells merely with a COX-2 inhibitor, nor could we detect any differences in COX-1 expression, suggesting a direct link

between BK-2R and COX-2 pathways in endothelial cells.

A connection between BK-2Rs and COX-2 has previously been suggested by Marceau *et al*. In this study diclofenac induced BK-2R down-regulation in rat hearts, although suspected, they could not show a feed-back mechanism between COX-2 and BK-2Rs (281).

Previous studies reported, that exogenous BK was able to induce COX-2 expression, in different cell types (55;344). Moreover, in rat aortic VSMC, BK induced the expression of COX-2, and the effect was reversed by icatibant and L-NAME, but not by a BK-1R inhibitor. (379). Also, in rabbits infusion of COX-2 inhibitor (diclofenac) induced BK-2R down-regulation in the rabbit heart (281). In a recent study, the positive post-conditioning effect of BK was abolished by a COX-inhibitor, which was thought to relate to decreased production of PGI_2 (349). COX-2 induction by BK can be blocked by icatibant or L-NAME, suggesting, that at least in ECs, the induction of COX-2, involves activation of BK-2 receptors and eNOS. Several studies show, that RhoGTPases are involved in the regulation of COX-2, although the exact mechanisms are still not clear.

Thus, it can be suggested that, in lovastatin treated endothelial cells, BK induces BK-2Rs, increasing the production of NO and PGI_2 via eNOS and COX-2, respectively, leading to increased vasodilation in the vasculature. Whether the COX-2 effect relates to increased prostacyclin production, to decreased production of contracting prostanoids or to some other mechanism, can not be addressed.

It can also be speculated, whether the negative cardiovascular effects of COX-2 inhibitors observed in large clinical studies, partly relates to the down-regulation of BK-2Rs.

Endothelial Hypoxia and Preconditioning

Hypoxia is a common factor in cardiovascular diseases, regardless of etiology. Hypoxia stimulates BK production and the expression of eNOS is known to increase in hypoxic conditions. Several studies focusing on the effects of BK in both animals and humans exists, but again the significance of BK-receptors in hypoxia is not conclusively studied. In our *in vitro* studies mild hypoxia induced functional BK-2Rs in hCAECs, but also in coronary artery smooth muscle cells (CASMC), and the effect was reversed by icatibant. This might give a mechanistical explanation for pre- and postconditioning, as BK is known to be one of the main effector molecules in preconditioning. In experimental models, short intervals of repeated hypoxia are known to be beneficial to the heart, improving the outcome after induced myocardial infarction (323). Postconditioning also augments the deleterious effects induced by reperfusion, and activation of BK-BK-2Rs is involved in this

cardioprotection (350). Hence, our results from hypoxic ECs, might reflect the protection seen in pre-and postconditioning. Indeed, the cardioprotective effects of preconditioning are abolished in BK-2R knockout mice and BK induces and icatibant attenuates the cardioprotective effects in a rabbit model of preconditioning (475). Moreover, in a recent study, postconditioning or intermittent BK infusion was shown to protect the myocardium and involve activation of COX-2 and subsequent PGI_2 formation (349).

We showed that hypoxia also induced migration of endothelial cells, which was abrogated by icatibant, suggesting a role for BK-2Rs in angiogenesis. This is consistent with previous results in which BK-2Rs were shown to transactivate VEGF receptors (KDR/Flk-1) and to induce tube formation in hCAECs (310). Similar results have been shown in mouse hearts, where angiotensin II and hypoxia significantly increased angiogenesis via AT-2Rs and the effects were BK-BK-2R and eNOS -dependent (322). Enalapril was also able to induce sprouting in hypoxic mouse hearts through both BK-1Rs and BK-2Rs, requiring the presence of both BK and BK-2Rs. Thus, BK and other vasoactive molecules can promote angiogenesis in hypoxic mouse hearts by activating BK-2Rs (384).

Our results showed that under hypoxic conditions, induction of BK-2Rs in cultured ECs was clearly attenuated by exogenous BK, which also increased the basal NO production.

Our results from rats, recovering from myocardial infarction, showed an induction of BK-2Rs in the endothelium of vessels forming at the border zone between the scar tissue and healthy myocardium, i.e. where potential ischemia augments BK formation and BK-2R expression and enhances angiogenesis. This further implies that the pathophysiological mechanisms activated during ischemic injuries also involve activation of the kinin receptor system.

Supporting our results are also the observations, that ACE inhibitor treatment or tissue kallikrein gene delivery increased myocardial capillary density and accelerated cardiac remodeling in SHRs in a BK-2R-dependent way (48;164).

Our results show that mild hypoxia induces the expression of functional BK-2Rs capable of increasing NO production and that BK-2Rs play a role in angiogenesis and EC migration in hypoxic conditions. There are also indications that PGI_2 via COX-2 induction might enhance the cardioprotective effects of kinin system.

In cardiovascular diseases, such as hypertension and CHD, which often lead to clinical HF, the cardioprotective role of BK-2Rs has been shown.

BK and BK-2Rs oppose endothelial dysfunction and contribute to angiogenesis(132;322). The kinin system decreases preload and cardiac output, thus improving myocardial metabolism and performance. BK also exerts a unique pattern of injury limiting actions in preconditioning (444;475). It exerts

cardioprotective effects when administered prior to the onset of ischemia, participates in early preconditioning, and is also an important factor in late preconditioning (235;402;468). ACE inhibitors and NEP inhibitors increase bradykinin concentration in the myocardium, thus they increase the protective effects of BK in ischemic diseases (325;446;483). Exogenous bradykinin, augmenting endogenous BK levels or activating BK-2Rs, possibly also BK-1Rs, could have an impact on the treatment of ischemic heart diseases.

From previous results hypothesis can be made that during the progression from compensated left ventricular hypertrophy to fulminate heart failure, the heart tries to compensate for ongoing changes by increasing the number of BK-2Rs, but in the end fails to do so and clinical HF develops, accompanied by diminished BK-2R levels and increased BK-1R levels(239;260). Is the diseased myocardium unable to react to BK and induce the BK-2Rs, in end-stage hearts, where the induced myocardial growth is accompanied with diminished NO production and unbalanced Ang II secretion. Or, are there other factors contributing to the down-regulation of BK-2Rs and progression of HF, can not be answered. Also, wheater the induction of BK-1Rs is a compensatory mechanism towards the down-regulation of BK-2Rs or a response to the inflammatory cascades activated, are not known.

The relevance of BK-1Rs in heart failure is still debatable. If the up-regulation of BK-1Rs in end-stage HF is cardioprotective, the induction of BK-1R could represent compensatory effect for BK-2R down-regulation. Several studies show, that BK-1Rs take over the protective effects of BK-2Rs, when the BK-2R are genetically or pharmacologically deleted. There are also several contradictory reports, were the induction of BK-1Rs accompanies activation of cardiotoxic pathways and progression of the cardiovascular disease.

Clinical Implications

Statin treatment has been shown to have several cholesterol-independent mechanisms that counteract endothelial function. Inhibition of the Rho-Rho kinase pathway partly accounts for the beneficial pleoitropic effects of statins and relates to the induction of BK-2Rs, thus improving the vascular endothelial dysfunction.

The beneficial cardiovascular effects of ACE inhibitor treatment are generally considered to arise from the prevention of BK degradation and the induction of BK-2Rs thus promoting vasodilation in endothelial cells, and inhibition of myocyte hypertrophy. Blockade of the BK-2R, partially prevents the cardiovascular benefits of ACE inhibition in different disease models, demonstrating the pharmacological relevance of bradykinin signaling in cardiovascular disease (263).

As BK in degraded by both ACE and NEP, simultaneous inhibition prevents the degradation more efficiently than ACE inhibition alone, leading to increased plasma and tissue concentrations of BK (40). The most extensively studied dual inhibitor, omapatrilat, has been studied in human heart failure, and it has shown superior results in HF when compared to ACE inhibitors. Unfortunately severe angioedema, in higher frequencies in patients receiving omapatrilat, has been reported and because of these serious concerns, it has not been approved for the treatment of HF (19).

Several chemical and physiological factors regulate the BK-R expression. Moreover, kinins are known to affect most pathological conditions leading to LVH and HF. Actions intensifying either BK and/or BK-2Rs, might give additional tools for the treatment of HF. The molecular mechanisms leading to HF are not clear, and among other pathways, the kinin system and factors regulating it, need more studies, in order to find therapeutical drugs for treating HF.

14. Summary and Conclusions

1. In the human end-stage heart failure functional BK-1Rs were significantly increased in the intra myocardial coronary vessels. This induction was accompanied by accumulation of TNF-α and the receptors co-localized with TNFR1 in the myocardial vessels, referring that the induced BK-1Rs relate to inflammation and increased fibrosis in heart failure. This implies that inflammation regulates the expression of BK-1Rs in human HF.

Because the known cardioprotective actions of BK are mediated via BK-2Rs, whereas BK-1Rs are induced by tissue damage, these results suggest that the decreased BK-2R levels in the end-stage HF, may leave the hearts vulnerable to damages, and increases in the BK-1R expression and activity may represent a compensatory reaction in the diseased hearts.

2. The expression of BR-2Rs increased with age in healthy human hearts, which suggests that the human heart adapts to age-related changes in heart, by up-regulating the expression of cardioprotective BK-2Rs.

From previously known polymorphisms, the 9bp insertion/deletion SNP didn't seem to have a role in the progression of HF. The C-allele of -58 T/C SNP was more abundant in the IDC patients, which may account for the lower BK-2R mRNA levels detected and the development of HF.

Our results also suggest that different signaling cascades are activated in CHD and IDC patients.

3. Lovastatin treatment clearly increased the amount of functional BK-2Rs in the endothelial cells, involving Rho-Rho kinase pathways, previously shown to have an impact on the progression of cardiovascular diseases. Thus, some of the beneficial cardiovascular effects of statins may relate to increased number of BK-2Rs in the endothelium.

A connection between COX-2 expression and BK-2R was also presented. Our results arose an intriguing question of whether the use of COX-inhibitors and the increased risk of myocardial infarction, relates to down-regulation of BK-2Rs.

Our results suggest that HF patients benefit from statin treatment also via induction of BK-2Rs and increased NO production, thus improving the endothelial function. We also suggest that by decreasing the level of BK-2Rs, COX-inhibitors promote dysfunction of the endothelium and together with increased thrombosis activation, may worsen the outcome of HF patients.

4. Mild hypoxia induced the expression of BK-Rs in cultured endothelial cells. Exogenous BK induced the expression of BK-2Rs and also the migration of endothelial cells in hypoxic conditions. This migration was abrogated by inhibiting the BK-2Rs, suggesting a role for BK-2Rs on EC migration and proliferation in hypoxic conditions. The angiogenesis promoting effects were also shown in an infarct model, where BK-2Rs were localized at the infarct border zone, where hypoxia likely exists. Hypoxia induces BK and BK-2R formation and NO production, which promote angiogenesis and the healing of the myocardium.

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